THE UNIVERSITY OF ALBERTA

VITAMIN B_6 REQUIREMENT OF YOUNG ADULT WOMEN USING ORAL CONTRACEPTIVES

BY

(C) THERISA K. HOLT

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

SCHOOL OF HOUSEHOLD ECONOMICS

EDMONTON, ALBERTA

SPRING, 1972

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled VITAMIN B_6 REQUIREMENT OF YOUNG ADULT WOMEN USING ORAL CONTRACEPTIVES submitted by THERISA K. HOLT in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Five healthy young women who had been taking oral contraceptives of the combined type for at least 6 months and five healthy young women who were not consuming such preparations participated in a study to investigate the effect of usage of oral contraceptive agents on vitamin B_6 nutriture. A diet of known composition was consumed for 6 days. The 6-day study period was planned to coincide with days 9 to 14 of the menstrual cycle of each subject. On the morning of day 6 of the study a fasting venous blood sample was taken followed by administration of a 2-gm L-tryptophan load dose. Throughout the 6-day study 24-hour urine collections were made and composited so that days 1-4 were pooled, whereas the day 5 and day 6 collections were considered to be the pre-load and post-load samples respectively. The urine was analyzed for tryptophan metabolites: xanthurenic acid, kynurenic acid and N'-methyl-nicotinamide, and for nitrogen.

The levels of the urinary tryptophan metabolites were not significantly different for either the control group or the experimental group when the days 1-4 composite was compared with the day 5 sample; however, following the tryptophan load a significant increase ($p < 0.05$) was noted in both the experimental and control groups for all tryptophan metabolites measured.

Comparison of the control group with the experimental group showed no significant difference in xanthurenic acid excretion for the composite made for days 1-4 whereas the experimental group excreted significantly greater ($p < 0.05$) amounts of xanthurenic acid on day 5 and day 6 than did the control group. Kynurenic acid excretion was

significantly increased ($P < 0.05$) in the control group compared with the experimental group for the days 1-4 composite, however, there was no significant difference between experimental and control groups on day 5 and day 6. The excretion of N^1-methylnicotinamide for the days 1-4 composite or day 5 was not significantly different for the experimental group compared to the control group, but a significantly greater ($P < 0.05$) excretion by the experimental group when compared to the control group occurred on day 6.

No significant differences in nitrogen excretion were noted either when pre-load and post-load values were compared or when the control group was compared with the experimental group thus indicating that the decreased levels of plasma amino acids in oral contraceptive users is not a result of urinary loss.

These results may indicate an increased requirement for vitamin B_6 in those taking oral contraceptives.

ACKNOWLEDGMENTS

The author wishes to express her appreciation for the direction in her studies provided by Dr. Elizabeth Donald. Thank you also to those who participated in the study, to Mrs. Barbara Hohol for her technical assistance, and to the author's husband for his encouragement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Vitamin B_6 supplementation may be of value in the correction of some of the biochemical and/or clinical disorders that have been reported to accompany the consumption of steroid contraceptive pills by women, thus an increased requirement for vitamin B_6 in users of such oral contraceptives may be indicated. A study of subjects using oral contraceptive pills and control subjects who are not undergoing such hormonal therapy may reveal some differences in tryptophan metabolite and nitrogen levels which are known to reflect vitamin B_6 requirement.

LITERATURE REVIEW

Since 1960, when the first oral contraceptive became available for general use, millions of women have used oral contraceptives as an effective and convenient method of family planning (1). Never before had a drug been used to inhibit a normal physiological process in the absence of disease. Recent concern as to the long term effects of the usage of oral contraceptives has led to human and animal studies investigating the possible metabolic alterations caused by such preparations.

In the 1968 publication of the National Research Council's Recommended Dietary Allowances (2) 2.0 mg of vitamin B_6 is stated as the daily allowance for normal adult women. Studies by Donald et al. (3) have indicated 1.5 mg of vitamin B_6 daily to be adequate for this same group. In contrast, recent studies conducted by Luhby et al. (4) indicated that 25 mg of pyridoxine daily was required to correct the altered vitamin B_6-tryptophan metabolism resulting from oral contraceptive use by 43 women. Allowing 5 mg as a safety factor, Luhby et al. have recommended 30 mg of vitamin B_6 daily for oral contraceptive users.

Oral contraceptives can be classified into two general categories, the combination type and the sequential type (5). Mixtures of both synthetic estrogens and progestogens taken daily over a period of 20 to 22 days in each menstrual cycle constitute the combination type. The sequential type attempts to more closely resemble natural hormonal production in that a synthetic estrogen is taken during the follicular phase of the cycle followed by a combination of both synthetic progestogen and estrogen in the luteal phase of the menstrual cycle. Follow-

ing cessation of medication after 20 to 22 days of administration of either type a simulated menstrual period occurs. The mode of action of oral contraceptives is not fully understood, but the result is clearly inhibition of ovulation.

Pyridoxal phosphate, the metabolically active form of vitamin B_6 (6), has been conclusively demonstrated to be essential in over 30 enzymatic reactions (2). Pyridoxal phosphate functions in fat and carbohydrate metabolism, however, its major metabolic function is in protein and amino acid metabolism (7).

Vitamin B_6 is essential for the metabolism of the amino acid tryptophan (8) (fig.1). Pronounced alterations in tryptophan metabolism are present in a vitamin B_6 deficiency. During a vitamin B_6 depletion study Yess et al. (9) periodically administered a 2-gm load dose of tryptophan to 6 male human subjects. They reported that urinary xanthurenic acid was elevated from a mean level of 41 μmoles/24 hours immediately before depletion began to 484 μmoles/24 hours at the height of depletion. Similarly, urinary kynurenic acid was increased from 54 to 88 μmoles, acetylkynurenine from 9 to 110 μmoles, kynurenine from 22 to 643 μmoles, and hydroxykynurenine from 21 to 1294 μmoles. Anthranilic acid glucuronide excretion decreased from 6 μmoles/24 hours to 5 μmoles/24 hours at the height of vitamin B_6 depletion as did o-aminohippuric acid from 48 to 31 μmoles daily. As a result of such findings the measurement of certain urinary tryptophan metabolites following a tryptophan load dose has been used widely as an index of vitamin B_6 nutriture (10). The 2-gm load dose of L-tryptophan has been recommended as this level seldom produces undesirable side effects which may occur at higher levels.

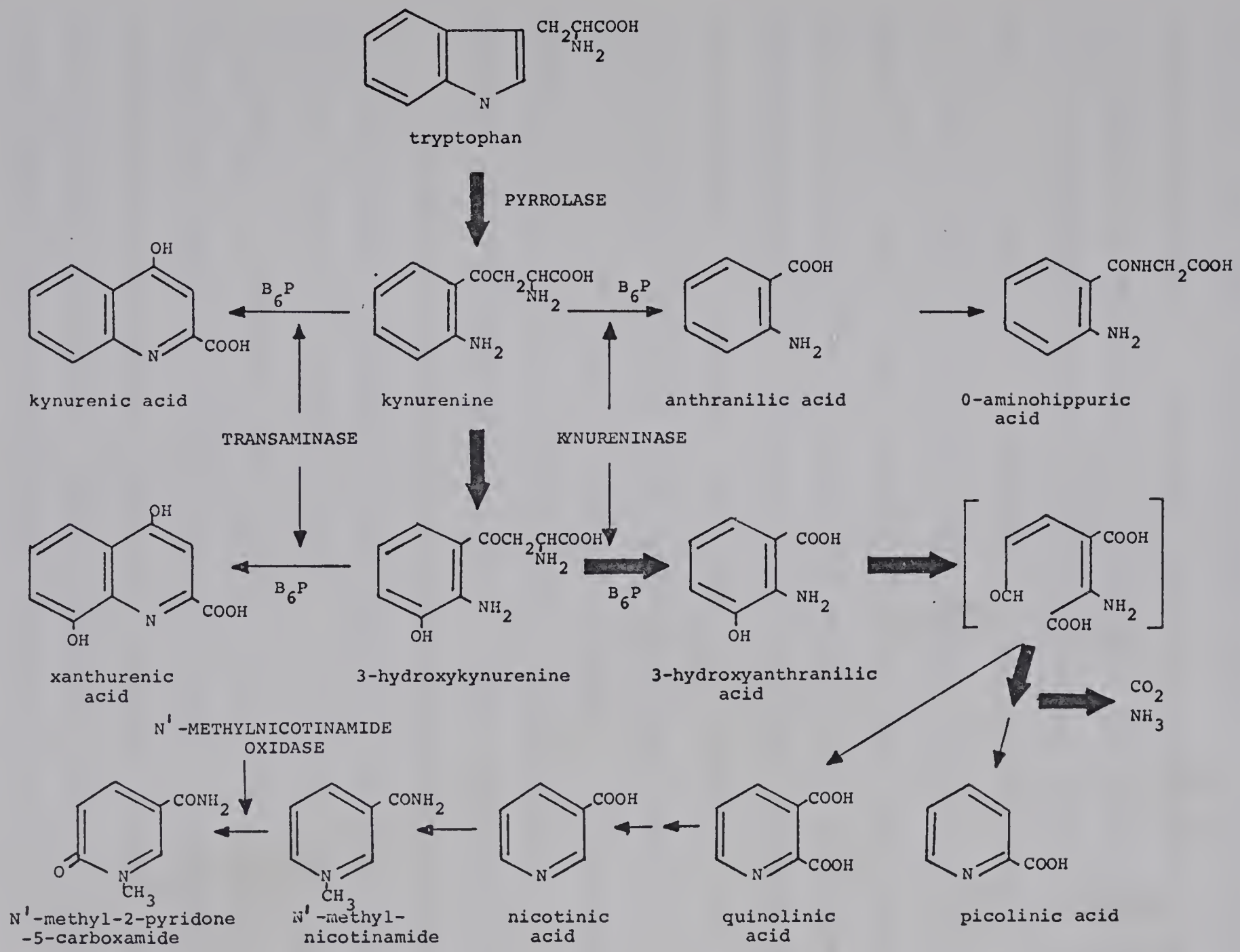

Figure 1 Pathway for the metabolism of tryptophan (8). The pathway believed to be of major significance on a quantitative basis is indicated by ➡.

In explanation of the changes which occur in tryptophan metabolism Ogasawara et al. (11) have reported that in vitamin B_6 deficiency the enzyme kynureninase becomes depleted of the coenzyme pyridoxal phosphate at a faster rate than does kynurenine transaminase thus resulting in an accumulation of kynurenine and 3-hydroxykynurenine and the transamination products, kynurenic acid and xanthurenic acid (fig.1). The elevation of xanthurenic acid has been historically used to indicate vitamin B_6 deficiency (12); however, Price et al. (13) have proposed that a more complete picture of the metabolic interrelationships can be obtained by the analysis of as many of the tryptophan metabolites as possible. Price et al. (14) have also suggested that the measurement of xanthurenic acid alone might not detect a more serious vitamin B_6 deficiency in which the apoenzyme kynurenine transaminase had lost the coenzyme, pyridoxal phosphate. Other tryptophan metabolites which serve as xanthurenic acid precursors, such as kynurenine and 3-hydroxykynurenine (fig.1), should also be determined.

Following the administration of oral contraceptive steroids abnormal tryptophan metabolism has been observed in humans in which vitamin B_6 intake was apparently adequate. In 1966, Rose (15) reported the excretion of xanthurenic acid ranging from 24 to 983 mg in the 8-hour urine collection following a 5-gm load dose of L-tryptophan in 14 women who had been taking a variety of progestogen-estrogen preparations for 1 month to 5 years. Twelve subjects consisting of 6 men and 6 women acted as controls and excreted a range of 10 to 60 mg of xanthurenic acid under similar conditions. After 5 days of oral administration of vitamin B_6 in the form of 40 mg of pyridoxine hydrochloride

daily to one experimental subject the urinary excretion of xanthurenic acid fell from 593 mg before vitamin B_6 administration to 80 mg after vitamin B_6 supplementation as measured in an 8-hour collection of urine following a 5-gm load dose of tryptophan. To explain such results Rose proposed that estrogens may have an inhibitory effect which interferes with the coenzyme function of pyridoxal-5-phosphate with the enzyme kynurenine transaminase.

Also in 1966, Rose (16) published the results of a study in which urinary xanthurenic acid as well as other tryptophan metabolites (Table 1) were measured in the 8-hour collection following a 5-gm L-tryptophan test dose. The subjects studied were 10 women taking a variety of oral contraceptives of the combination type, 5 who were taking estrogen therapy for haemorrhagic disorders, and 22 healthy control subjects not undergoing hormonal therapy. Xanthurenic acid levels were elevated in the hormone-treated groups which closely agreed with the previous work of the same author. Twenty milligrams of pyridoxine hydrochloride was administered orally twice daily for 5 days to one of the subjects taking oral contraceptives with the result that xanthurenic acid excretion decreased from 2688 μmoles prior to vitamin B_6 administration to 336 μmoles following supplementation in the 8-hour collection following the load dose. Administration of a single 20-mg dose of pyridoxine hydrochloride with the tryptophan load dose to one of the estrogen-treated subjects likewise produced a post-tryptophan reduction of xanthurenic acid from 662 μmoles before pyridoxine administration to 87 μmoles following supplementation. The administration of vitamin B_6 in large doses thus lowered the previously high excretion

Table 1

Average urinary excretion of tryptophan metabolites[1] following a 5-gm load dose of L-tryptophan. Rose (16)

Subjects	XA[2]	HK[2]	HA[2]
Control	135	266	119
Estrogen-progestogen	1874	1525	599
Estrogen	854	573	454

1 XA = xanthurenic acid.

HK = 3-hydroxykynurenine.

HA = 3-hydroxyanthranilic acid.

2 Values are expressed as μmoles/8 hours.

of xanthurenic acid in the hormone-treated group to levels near that of the control group.

Price et al. (17) conducted a study similar to that of Rose (16). Ten young women ingesting oral contraceptive pills containing 2.5 mg of norethynodrel (progestogen) and 0.1 mg of mestranol (estrogen) were compared to 18 healthy female control subjects not taking these drugs. Urinary excretion levels of tryptophan metabolites before and after ingestion of a 2-gm load dose of L-tryptophan were measured. The mean xanthurenic acid excretion levels prior to the tryptophan load dose differed by less than 2 standard deviations in the control and experimental groups (Table 2). After administration of the loading dose of tryptophan the subjects taking the oral contraceptives excreted a mean level of 697 μmoles/24 hours of xanthurenic acid which was greater than 2 standard deviations above the mean level of 30 μmoles/24 hours excreted by the control group (Table 2). Pyridoxine supplementation to the experimental group in the form of 25 mg of pyridoxine hydrochloride 4 times daily for 2 days resulted in the excretion of levels of xanthurenic acid by the experimental group which were within the range of the control group (Table 2).

The levels of urinary xanthurenic acid in the study of Price et al. (17) were somewhat lower than in the previous work of Rose (16) because Price used a 2-gm load dose of tryptophan rather than the 5-gm dose which Rose had used.

To study the metabolic effect of oral steroid hormones Aly and coworkers (18) conducted a 6-day controlled diet study with an experimental group of 5 young women who had been taking a variety of oral

Table 2

Average urinary excretion of tryptophan metabolites[1] by 10 subjects ingesting oral contraceptives and 18 control subjects. Price et al. (17)

Subjects	Treatment		XA[4]	KYN[4]	KA[4]	HKYN[4]	AAG[4]	O-AH[4]	PYR[4]
	Tryptophan[2]	Pyridoxine[3]							
Control	---	---	8	10	13	21	4	21	89
Experimental	---	---	11	10	12	17	5	18	64
Experimental	---	200	13*	11	13	17	6	20	121
Control	2.0	---	30	28	60	43	7	40	131
Experimental	2.0	---	697*	171*	102*	250*	12	24	136
Experimental	2.0	200	42	22	39	26	12*	29	185

1 The following abbreviations are used: XA, xanthurenic acid; KYN, kynurenine; KA, kynurenic acid; HKYN, 3-hydroxykynurenine; AAG, anthranilic acid glucuronide; O-AH, O-aminohippuric acid; and PYR, N-methyl-5-carboxamide (pyridone).

2 Values are expressed in grams.

3 Values are expressed in milligrams.

4 Values are expressed as μmoles/24 hours.

* Indicates values differ from the mean values for controls by more than 2 standard deviations.

contraceptive pills of the combined type for 11 to 50 months and a control group of 5 women who had not been using such hormones. Xanthurenic acid (Table 3), as well as other tryptophan metabolites, were measured in the 24-hour collection of urine prior to and following a 2-gm oral dose of L-tryptophan. Little difference was observed between the pre-load xanthurenic acid levels of the two groups; however, the differences between control and experimental groups following tryptophan loading was significant at a $P < 0.001$. The findings of this study agree with that of Price et al. (17) although Price reported a greater mean increase in xanthurenic acid excretion after the administration of a tryptophan load dose in subjects using oral contraceptives.

In a recent study, Luhby et al. (4) attempted to determine the minimum daily oral dose of pyridoxine hydrochloride necessary to decrease urinary xanthurenic acid levels in an experimental group of 33 female subjects taking oral contraceptives to levels near that of a control group of 10 women. The experimental group had been using a variety of oral contraceptive pills for 3 months to 6 years. Each subject participated in the study for 3 consecutive menstrual cycles. During this time they were each given a 2-gm L-tryptophan load near the beginning, at the mid point, and at the end of each menstrual cycle. Urine was collected for the 8 hours immediately following the tryptophan load. Pyridoxine hydrochloride was administered to some of the experimental and control subjects at a daily dosage of 2, 5, 10, or 20 mg levels. Each level of pyridoxine hydrochloride was administered for one complete menstrual cycle. Five of the users of oral contraceptives and 8 non-users were not given pyridoxine supplementation.

Table 3

Average urinary excretion of tryptophan metabolites[1] by 5 women using oral contraceptives and 5 controls before and after a 2-gm load dose of L-tryptophan. Aly et al. (18)

Treatment	XA[2]	KYN[2]	KA[2]	HKYN[2]
Basal				
Control	25	2	17	29
Experimental	30*	7*	14*	42
Post-tryptophan				
Control	83	25	56	40
Experimental	293*	20	56	50

1 The following abbreviations are used: XA, xanthurenic acid; KYN, kynurenine; KA, kynurenic acid; and HKYN, 3-hydroxykynurenine.

2 Values are expressed as μmoles/24 hours.

* Indicates values which differ significantly from the mean values for controls according to the Student's t-test at a $P < 0.05$.

The mean individual xanthurenic acid excretion levels in the urine of the control group ranged from 9 to 18 μmoles/8 hours. None of the control group excreted greater than 35 μmoles of xanthurenic acid/8 hours; therefore, excretions in excess of 35 μmoles/8 hours were considered to be abnormal under these test conditions.

Approximately three-quarters of the experimental group excreted more than 35 μmoles of xanthurenic acid/8 hours during one or more of the test periods. Among the 33 experimental subjects the maximum excretion following the tryptophan loading tests ranged from 20 to 663 μmoles/8 hours. The mean excretion was 167 μmoles/8 hours.

The 2-mg dose of pyridoxine hydrochloride, equivalent to the 1968 NRC-Recommended Dietary Allowance (2) for vitamin B_6, was sufficient to decrease xanthurenic acid excretion following a 2-gm load dose of L-tryptophan to less than 35 μmoles/8 hours in only 10% of the oral contraceptive users; whereas, a 5-mg dose still resulted in xanthurenic acid levels greater than 35 μmoles/8 hours in 1/3 of the experimental group. Following the administration of 10 mg of pyridoxine hydrochloride daily 25% of the experimental group continued to produce more than 35 μmoles/8 hours and even after supplementation with 20 mg of pyridoxine hydrochloride 14% of those using estrogen-progestogen agents excreted an excess of xanthurenic acid. Using regression analysis of the dose-response data Luhby et al. (4) calculated that 25 mg of pyridoxine hydrochloride daily would be required to decrease xanthurenic acid excretion to less than 35 μmoles/8 hours in "all" subjects taking oral contraceptives. In view of the limited numbers of subjects studied and allowing 5 mg as a safety factor Luhby et al. have recom-

mended a daily allowance of 30 mg of pyridoxine hydrochloride for women using oral contraceptives.

Urinary kynurenine and kynurenic acid levels in subjects taking oral contraceptives compared to a control group were reported by Price and associates (17) and by Aly and coworkers (18). Price et al. found that steroid hormones had no significant effect on the excretion of kynurenine and kynurenic acid previous to the administration of tryptophan (Table 2); however, post-load excretion levels of both metabolites in the experimental group were more than 2 standard deviations above the mean value for the control group. Vitamin B_6 administration of 100 mg/day of pyridoxine hydrochloride for 2 days to the experimental subjects decreased post-tryptophan kynurenine and kynurenic acid excretion to levels similar to that excreted by the control group.

The results of a study by Aly and coworkers (Table 3) were somewhat different than those of Price et al. Prior to the administration of a load dose of tryptophan, kynurenine excretion was significantly greater in the experimental group than the control group ($P<0.02$), however, kynurenic acid excretion was significantly greater ($P<0.05$) in the control group compared to the experimental group. Following loading no statistically significant difference between groups was noted for either kynurenine or kynurenic acid production although values reported for the experimental group were much lower than those reported by Price et al. (17). Rose (16) also measured urinary 3-hydroxykynurenine levels in the 8-hour collection following a 5-gm load dose of L-tryptophan (Table 1). Elevated levels of 3-hydroxykynurenine were found in the estrogen-progestogen treated subjects and in subjects

receiving only estrogen compared to a control group. Administration of 20 mg of pyridoxine hydrochloride the night before a 5-gm tryptophan load plus 20 mg of pyridoxine hydrochloride with the tryptophan load to one subject taking estrogen-progestogen preparations reduced the 3-hydroxykynurenine excretion from 2101 μmoles/8 hours prior to pyridoxine administration to 121 μmoles/8 hours following vitamin supplementation. Similarly, 3-hydroxykynurenine excretion was decreased from 423 μmoles prior to supplementation to 54 μmoles by the addition of 20 mg of pyridoxine hydrochloride to the tryptophan load of one estrogen-treated subject.

Rose (16) suggested that his findings in this experiment indicated an estrogen-induced increase in the capacity for conversion of tryptophan to nicotinic acid. As a result, tryptophan loading produced a relative shortage of pyridoxal phosphate coenzyme and a consequent increase in metabolites of tryptophan.

Price et al. (17) found a comparable trend in 3-hydroxykynurenine excretion in 24-hour collections prior to and following a 2-gm load dose of tryptophan (Table 2). No difference between the control and the experimental group existed before loading, but following the tryptophan load a statistically significantly greater amount of 3-hydroxykynurenine was excreted by the experimental group compared to the control group. Administration of 100 mg of pyridoxine hydrochloride daily for 2 days to the experimental group resulted in excretion of 3-hydroxykynurenine which was within the range of the control group both before and after tryptophan loading (Table 2).

In contrast, Aly and coworkers (18) found no significant difference in 3-hydroxykynurenine excretion in a control group and in subjects using oral contraceptives before and after a 2-gm load dose of L-tryptophan (Table 3).

Price et al. (17) reported urinary anthranilic acid (fig.1) levels determined as the glucuronide in the 24-hour collection of a group of young women using oral contraceptives and a control group before and after a 2-gm load dose of L-tryptophan. Those using oral contraceptives excreted essentially the same amount of anthranilic acid glucuronide as the control group before and after loading (Table 2). The administration of 25 mg of pyridoxine hydrochloride 4 times daily for 2 days to the experimental group produced little pre-load change in the excretion of anthranilic acid glucuronide; however, following the load the experimental group excreted 12 μmoles of anthranilic acid glucuronide which was greater than 2 standard deviations above that of the control group.

Price et al. (17) also determined o-aminohippuric acid (fig.1) and found no significant difference in the excretion between oral contraceptive users and a control group before and after pyridoxine supplementation (Table 2). In each case measurements were made prior to and following a 2-gm load dose of L-tryptophan.

Rose (16) reported that in some, but not all, users of oral contraceptives 3-hydroxyanthranilic acid levels were elevated. The mean level of 3-hydroxyanthranilic acid excreted by the control group was much lower than the estrogen-progestogen subjects or the estrogen therapy subjects (Table 1). The administration of 20 mg of pyridoxine

hydrochloride the night prior to the load dose of tryptophan plus 20 mg of pyridoxine hydrochloride with the load dose to one oral contraceptive user reduced the 3-hydroxyanthranilic acid excretion from 578 μmoles in the 8-hour collection following the load before vitamin supplementation to 234 μmoles following supplementation. Similarly, 20 mg of pyridoxine hydrochloride with the load to one of the estrogen-treated subjects decreased the 3-hydroxyanthranilic acid level from 136 μmoles before the vitamin administration to 45 μmoles following supplementation.

Rose (16) noted that since 3-hydroxyanthranilic acid is the result of the kynureninase action on 3-hydroxykynurenine (fig.1) elevation of 3-hydroxyanthranilic acid would not normally be expected in a simple vitamin B_6 deficiency in which kynureninase activity is depressed. The fact that pyridoxine administration lowered the elevated levels of 3-hydroxyanthranilic acid in the hormone-treated subjects suggested that vitamin B_6 is involved in one or more enzymatic steps beyond 3-hydroxyanthranilic acid.

Rose et al. (19) extended the study of Price et al. (17) by examining the urinary excretion of N^1-methylnicotinamide (fig.1) as well as 2-pyridone (N^1-methyl-2-pyridone-5-carboxamide) in 10 women using oral contraceptives before and after a 2-gm load dose of L-tryptophan. The results were compared with those of a healthy control group of premenopausal women. Rose et al. (19) found that the experimental group excreted 60 μmoles/24 hours of N^1-methylnicotinamide prior to the load which was significantly more than the 32 μmoles/24 hours produced by the control group under similar conditions. Following loading 148 μmoles/24 hours of N^1-methylnicotinamide was produced by the oral

contraceptive users compared to the significantly lower 75 μmoles excreted by the control group. Pyridoxine hydrochloride given as 25 mg 4 times daily for 2 days to the experimental and control groups increased urinary N'-methylnicotinamide to 102 μmoles/24 hours in the experimental group and 52 μmoles in the control group before the load dose, and to 184 μmoles in the oral contraceptive group and 94 μmoles in the control group following loading. Both pre- and post-tryptophan values for the experimental group differed statistically from those of the control group according to the Student's t-test ($P < 0.05$). The administration of pyridoxine therefore did not decrease N'-methylnicotinamide in the experimental group to values within the range of the control group as had been the case with some of the other tryptophan metabolites (17).

Pre-tryptophan excretion of 2-pyridone in the experimental group in this same study by Rose et al. was 64 μmoles/24 hours which was not significantly different from the 86 μmoles produced by the control group. Following the tryptophan load dose 136 μmoles were produced by the oral contraceptive users and 140 μmoles/24 hours by the control group. Pyridoxine supplementation resulted in a pre-tryptophan load increase in the experimental group to 121 μmoles/24 hours and to 149 μmoles in the control group. Post-tryptophan excretion was 185 μmoles in the experimental group and 191 μmoles in the control group. No significant difference in 2-pyridone excretion between the control and the experimental group was noted.

Price et al. (17) had previously reported 2-pyridone excretion in oral contraceptive users and a control group (Table 2). The results

obtained from the experimental group were similar to those of Rose et al. (19). The control group, however, in the study of Price et al. did not undergo pyridoxine supplementation as in the group studied by Rose et al., and as a result the control group excreted only 89 μmoles of 2-pyridone before the load and 131 μmoles following loading. Nevertheless, no significant difference in 2-pyridone excretion between the experimental and control group was found.

Rose et al. (19) have explained these results by suggesting both a hormone-mediated increase in the conversion of tryptophan to N^1-methylnicotinamide plus a partial interference with the subsequent metabolism of N^1-methylnicotinamide to 2-pyridone (fig.1). Studies conducted by Glüecksohn-Waelsch et al. (20) with mice are consistent with this explanation as they found that estradiol administration reduced the activity of the enzyme which catalyzes the oxidation of N^1-methylnicotinamide to 2-pyridone by 60% in one experiment and by 76% in another.

In addition to the altered tryptophan metabolism in subjects using oral contraceptives, differences in plasma and urinary nitrogen have been reported in oral contraceptive users compared to a control group.

Aly and coworkers (18) found that the total plasma free amino acid levels were significantly lower in subjects taking oral contraceptives ($P < 0.02$) due to a marked decrease in the level of some of the non-essential amino acids. The total for the six essential amino acids determined was 78 μmoles/100 ml of plasma for the experimental subjects. This difference was not significant when compared with the levels found in the control group. Nine non-essential amino acids were measured and

a total of 133 μmoles/100 ml plasma was detected in the control group compared with 101 μmoles/100 ml of plasma for the experimental group. This difference was significant at a $P < 0.02$.

Aly et al. (18) suggest that the decrease in plasma levels of some amino acids in the experimental group could perhaps be explained by a shift of these amino acids to some other tissue in the body or to a possible increase in their urinary excretion.

Craft and Wise (21) reported lower levels of fasting plasma L-amino nitrogen in subjects taking oral contraceptives compared with a control group. Experimental subjects had 3.31 mg of amino nitrogen/ 100 ml plasma compared with 3.64 mg for the control group. For plasma L-amino nitrogen the ratio of experimental to control group was 0.90 in the study of Craft and Wise (21) compared with 0.83 for the amino acids measured in the study of Aly et al. (18).

Craft and Wise (21) also noted that in normal women there was a tendency for the fasting level of plasma L-amino nitrogen to be lower in the second half of the menstrual cycle which is progesterone dominated, although not as low as levels found in users of oral contraceptives. They, therefore, offered in possible explanation for their results that synthetic progestogen is mildly anabolic and that there may be increased utilization of amino acids by the liver and peripheral tissues in those taking oral contraceptives.

Friedberg and Greenberg (22) also found that the administration of estrogen produced a significant lowering of the free plasma amino acid levels in male rats. A mean level of 6.4 mg of free amino acids/ 100 ml of plasma was reported in the control rats compared to 5.2 mg/

100 ml in the estrogen-treated rats. The difference was significant at $P < 0.02$.

In contrast, Zinneman and associates (23) reported that the administration of 5 mg of diethylstilbestrol (a synthetic estrogen) daily for 5 days to human male subjects produced no significant differences in free plasma and urinary amino acid levels.

In a later study Zinneman and coworkers (24) analyzed plasma and urine for 21 free amino acids in 5 males who had received 100 mg daily of progesterone intramuscularly for 7 days. After a 4-month interval this same group received 5 mg of diethylstilbestrol (a synthetic estrogen) daily combined with 100 mg daily of progesterone intramuscularly for 7 days. Although significant differences were noted for some amino acids there was no overall significant difference in total urinary and plasma amino acid levels following progesterone administration. An increase in urinary excretion of free amino acids was noted following progesterone combined with estrogen administration, whereas, the free plasma amino acid levels decreased but did not follow the same pattern as the urinary excretion.

In possible accord with the proposal that amino acids shift from the plasma to the tissues rather than to the urine, McCorquodale and Mueller (25) noted induction of rapid uterine growth in rats by estradiol (a synthetic estrogen).

EXPERIMENTAL PROCEDURE

Subjects

Ten subjects participated in the study. Five young women using oral contraceptive pills of the combined type made up the experimental group while another five women, not on hormonal therapy but having a history of regular menstrual cycles, served as controls. The chosen experimental group had been using steroids for at least six months before commencing the study because almost all reported adverse clinical symptoms or biochemical changes associated with such hormonal use are manifested within the first 3 months after commencing therapy (26). The names, dosage levels, and potencies of the oral contraceptives used as well as the duration of usage prior to the study are shown in Table 4. Several of the experimental subjects had experienced side effects which could possibly be attributed to pill usage. Reported side effects were nausea occurring on the first day of the cycle and melasma reported by subject 6, occasional spotting reported by subject 7, and a slight weight gain in subject 10.

A preliminary questionnaire followed by a pre-study clinical examination was used to eliminate unsuitable subjects so that all chosen volunteers were apparently healthy. Some general characteristics of the subjects are given in Table 5. No significant difference existed between the experimental and control groups in any of the characteristics noted using the unpaired t-test.

Table 4

Names and dosages of the oral contraceptive pills used by the experimental group in the present study

Subject	Commercial Name	PROGESTOGEN			ESTROGEN			Duration of Therapy[4]
		Name	Dosage Amount[1]	Potency[2]	Name	Dosage Amount[1]	Potency[3]	
6	Ovulen-1	ethynodiol diacetate	1.0	15.0	mestranol	0.10	2.0	27
7	Ortho-novum 1	norethindrone	1.0	1.0	mestranol	0.05	1.0	6*
8	Demulen	ethynodiol diacetate	1.0	15.0	ethinyl estradiol	0.05	1.8	28**
9	Ortho-novum .5	norethindrone	0.5	0.5	mestranol	0.10	2.0	13
10	Ortho-novum 1	norethindrone	1.0	1.0	mestranol	0.05	1.0	15

1 Values expressed as mg.

2 Relative to 1.0 mg of norethindrone with an assigned value of 1.0.

3 Relative to 0.05 mg of mestranol with an assigned value of 1.0.

4 Values expressed in months previous to commencement of the study.

* Subject 7 was on hormonal therapy for 56 months, had a successful pregnancy, and then restarted usage 6 months previous to beginning the study.

** Demulen used for only one month. Previously Ovulen-1 was taken.

Table 5

General characteristics of the subjects in the present study

Subject	Age[1]	Height[2]	Weight[3]	Menstrual cycle: Age of onset[4]	Menstrual cycle: Length of cycle[5]	Menstrual cycle: Length of bleeding[5]
Control Group						
1	23.0	5-0	114	13	32	4
2	26.5	5-6	127	12	30	5
3	23.0	5-3	124	13	28	3
4	24.0	5-6	117	13	28	3
5	23.0	5-5	135	13	28	5
Mean Value	24.0	5-4	123	13	30	4
Experimental Group						
6	24.5	5-8	161	13	28	6
7	28.0	5-4	120	14	28	4
8	26.0	5-2	118	13	28	3
9	25.0	5-2	111	12	28	4
10	22.5	5-4	126	15	28	3
Mean Value	25.0	5-4	127	13	28	4

1 Values are expressed in years to the nearest six months.

2 Values are expressed in feet plus inches without shoes.

3 Values are expressed to the nearest pound as recorded at the beginning of the study period.

4 Values are expressed in years.

5 Values are expressed in days.

The Study Period

To obtain information about the nutritional background of each volunteer a dietary record for the 3 days just prior to the study was recorded by each subject.

To standardize the dietary intake and therefore minimize the effect of diet on the indices to be measured the subjects were placed on a controlled dietary regime (Appendix I). The diet was such that it could be packaged so that it did not have to be consumed in any particular location.

The basal diet was consumed in entirety daily and met the National Research Council's 1968 Recommended Dietary Allowances (2) in all nutrients except iron. The calculated iron intake was 12.4 mg daily. Iron supplementation was not provided due to the short duration of the study and also because this level of iron met the Canadian dietary standard (27) of 10 mg daily for this group.

To allow for individual variation in caloric requirement an ad libitum source of additional energy was provided and consumed as desired (Appendix I). The kind and quantity of ad libitum consumption, any unusual activities, and the intake of any drugs were recorded by the subjects.

The study lasted 6 days. The first 4 days of the study allowed adjustment to the experimental diet. A 2-gm oral load dose of L-tryptophan was given in synthetic orange juice the morning of the sixth day. Day 5 was therefore considered as the pre-load or basal day and day 6 as the post-load day.

Timing of the Study Period

The timing of the metabolic study was particularly important since Rose (28) had shown that in the normal menstrual cycle elevated levels of tryptophan metabolites in the urine occurred following a 5-gm load dose of L-tryptophan given at the time of ovulation when compared to the immediate post-menstrual period. Likewise, Craft and Wise (21) found that the different phases of the menstrual cycle influenced fasting levels of plasma L-amino nitrogen.

Most of the physical complications reported to be associated with the usage of oral contraceptive pills are correlated with the estrogen content of the pill (26); therefore, the present study was so timed to coincide with the period of maximum estrogen secretion in the control group which is thought to occur at the point of ovulation (29) or the approximate midpoint of the menstrual cycle. The control group was chosen to have regular menstrual cycles, and as the experimental group had hormonally controlled cyclical activity, day 14 of the menstrual cycle was chosen as the most appropriate time for the administration of the tryptophan load dose. This was timed to occur on day 6 of the study.

In a normal menstrual cycle the basal body temperature is theoretically of a diphasic character due to the two phases of the cycle (29). Ovulation is associated with a drop in body temperature followed by an upward shift to a higher level. Immediately upon waking each day control subjects registered their oral basal body temperature beginning on the second day of the bleeding period and continuing through the experimental study until day 20 of the menstrual cycle.

This was used as an aid in determining whether the calculated ovulation time coincided with actual ovulation. Subjects taking oral contraceptive pills were not requested to register their basal body temperatures because ovulation in these subjects was suppressed (5).

Collection of Samples

a) Urine collection

Urine was collected in bottles containing a few crystals of thymol to serve as a preservative. On the morning of the ninth day of the menstrual cycle each subject completely emptied her bladder, discarded the urine, and then made continuous 24-hour collections for the next 6 days. The 24-hour urine collections were composited daily, the daily volume measured, and an aliquot was kept at -20^{o}C until analyzed. For the estimation of urinary tryptophan metabolites and nitrogen a pooled sample of urine was used from the first 4 days, and a separate sample from day 5 (pre-tryptophan) and day 6 (post-tryptophan) (Appendix II).

Since the body excretes a nearly constant amount of creatinine daily in the urine (30), the determination of creatinine was originally intended to be done daily and was to act as a check for completeness of daily urinary collections. Delay in receiving the creatinine standard necessitated freezing the samples for later analysis. Freezing in some way interfered with the determination when done by Folin's method cited by Oser (31) so that results could not be duplicated using this method. The method proposed for use with the B&L Spectronic 20 instrument produced satisfactory results (32). The standard curve obtained using this method appears in Figure 2. The amount of creatinine produced was

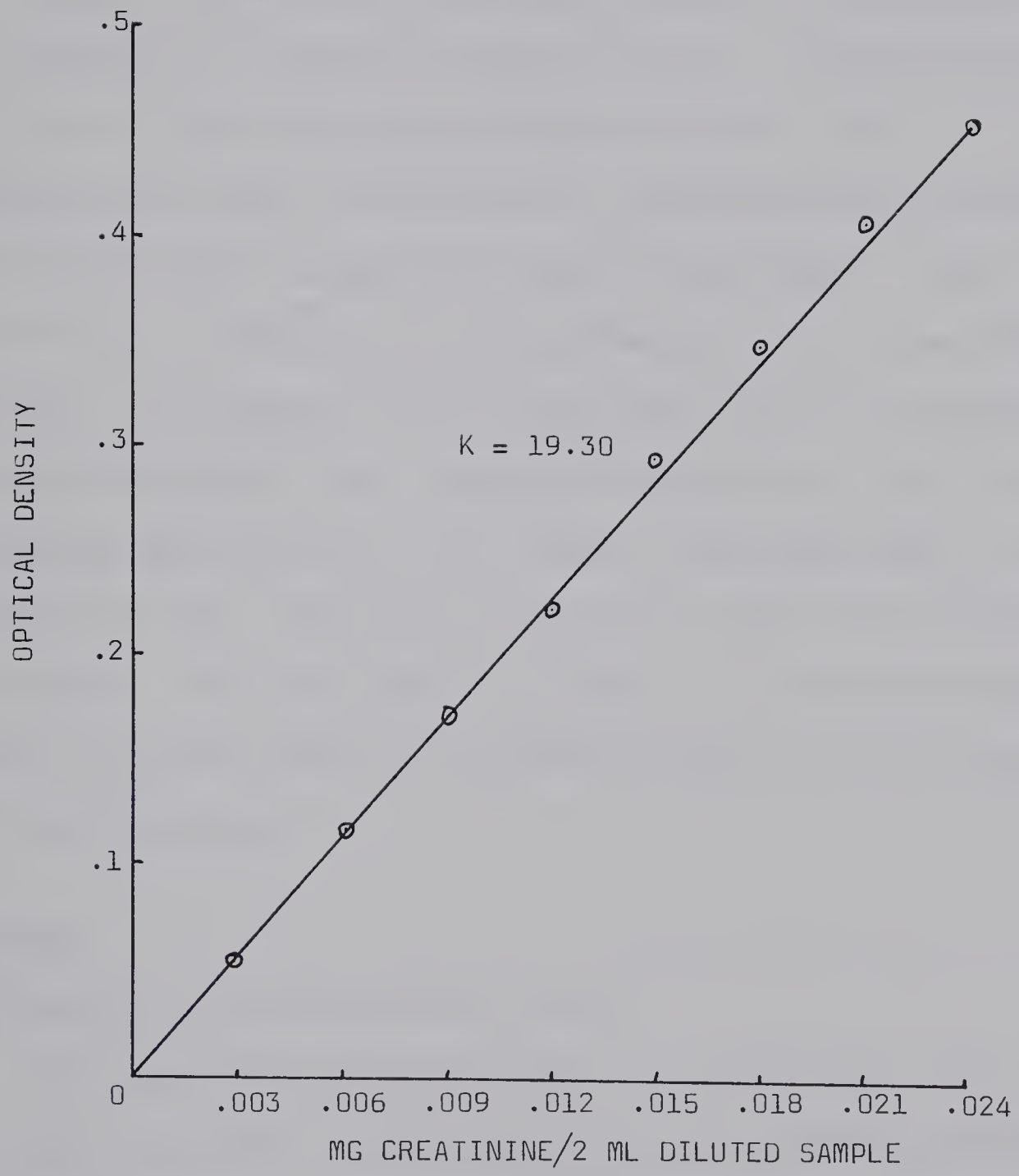

Figure 2 Creatinine standard curve.

calculated as: gm creatinine/24 hours =

$$\frac{\text{O.D. of sample X 24 hour urine volume(ml) X urine dilution factor X 5}}{\text{O.D. of creatinine standard/concentration of creatinine standard X 1000 (mg/2ml sample)}}$$

b) Blood samples

Several studies (33,34) have shown changes in the levels of some blood constituents at different times in the day. To avoid discrepancies due to this all blood sampling was done from 7:30 to 8:30 a.m. Ten ml of fasting venous blood was withdrawn by venapuncture before breakfast of day 6 of the study. Immediately the blood was mixed by gentle inversion with 2 mg of sodium ethylenediamine-tetracetic acid (Na-EDTA) per ml of blood. Two samples of whole blood were used for microhematocrit determination (Appendix III). Hemoglobin determinations were done by the oxyhemoglobin method (35). All tubes of blood were kept in crushed ice when not in use. The plasma and red blood cells were separated by centrifugation. The plasma was to be used for the future determination of amino acid levels whereas the vitamin B_6 level was to be determined in the red blood cells.

Methodology

a) Xanthurenic and kynurenic acid in urine

The fluorometric method of Price and coworkers (13), with minor modifications, was used for the determination of kynurenic and xanthurenic acid in the urine (Appendix IV). This procedure is based upon the principle described by Price and Dodge (36) that when normal human urine is passed through a column of Dowex 50 (H^+), kynurenic and xanthurenic acids remain on the ion exchange resin. Washing the resin

with a large volume of water results in the release of both acids from the column. Very few other substances are released during the treatment and those which are released are of minimal quantity and interference. Kynurenic acid exhibits maximal fluorescence in very strong acid, particularly in the presence of sulfuric acid, in contrast to alkali where it fluoreses minimally; whereas, xanthurenic acid fluoreses strongly in alkali and exhibits minimal fluorescence in very strong acid. A G. K. Turner fluorometer (Model 111) was used to determine fluorescence rather than the Coleman Model 12C photofluorometer used by Price and coworkers (13). This instrument was extremely sensitive and therefore an emission prism was removed and a 1% neutral density filter was inserted along with the other secondary filters to decrease the sensitivity. The optimal wavelengths for reading kynurenic acid fluorescence, according to Price and coworkers (13), are 340 mμ and 435 mμ for excitation and emission respectively. The primary and secondary filters used to achieve this were Corning numbers 7-60 and 3-75 respectively. For xanthurenic acid the primary and secondary filters used were Corning numbers 7-51 and 3-73 which correspond to the optimal xanthurenic acid fluorescence wavelengths of 370 mμ and 530 mμ respectively.

Four chromatographic columns were required for the determination of urinary xanthurenic acid and kynurenic acid for each subject as samples of the day 1 to 4 composite, day 5, and day 6 were analyzed as well as a sample for determining recovery on the day 1-4 composite. The urine from 2 subjects could thus be analyzed at one time because 8 chromatographic columns were handled at once.

b) N^1-methylnicotinamide in urine

N^1-methylnicotinamide in urine samples was determined using the method of Pelletier and Campbell (37) with minor modifications (Appendix V). This method is based on the formation of a stable fluorescent condensation product when N^1-methylnicotinamide is treated with ketone and alkali in the cold followed by heating in an acid medium (38).

Cold urea is initially introduced into the urine sample to protect the amide group of N^1-methylnicotinamide during the subsequent treatment with NaOH thus increasing the fluorescence. The urea must be kept cold following preparation to prevent the formation of ammonia which also produces a decrease in the fluorescence of urine samples as amide groups would not be protected. An excess of ketone is then added followed by the addition of NaOH, then HCl, and buffer, and finally the tubes are heated in a water bath at $85^{o}C$ for 4 minutes. Any interfering pigments produced are extracted into the upper ketone layer following saturation of this layer with buffer. Samples of the lower layer containing the condensation product of N^1-methylnicotinamide are removed for the determination of fluorescence.

A typical standard curve for N^1-methylnicotinamide obtained using this method is shown in Figure 3.

c) Urinary nitrogen

Urinary nitrogen analyses were conducted on the day 1-4 composite, day 5, and day 6 collections. The macro-Kjeldahl A.O.A.C. method (39) was used with some variations (Appendix VI).

Theoretically, this method involves the oxidation of nitrogenous substances in a urine sample by boiling with concentrated sulfuric acid

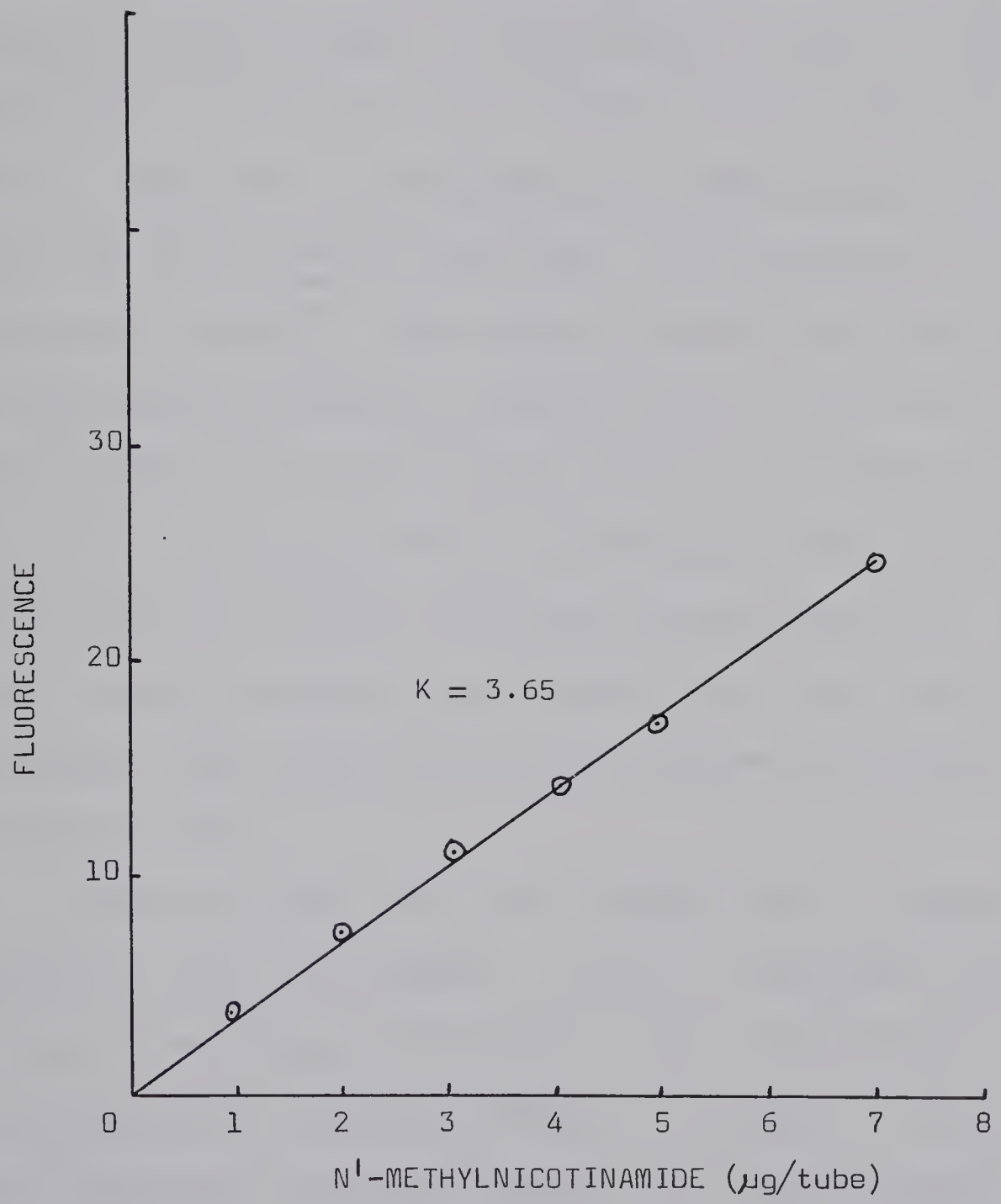

Figure 3 Standard curve for N'-methylnicotinamide.

in a macro-Kjeldahl flask. The nitrogen is converted to ammonia and is fixed as ammonium sulfate. A Kel-Pak[1] containing 9.9 gm K_2SO_4, 0.41 gm HgO and 0.08 gm $CuSO_4$ is added to the urine-acid mixture and acts as a catalyst in this conversion. $CuSO_4$ and HgO lower the temperature at which the urinary nitrogen is converted to ammonium sulfate while K_2SO_4 increases the boiling point of the urine-acid solution. This mixture is digested for 35 minutes after the solution becomes clear green. The flasks are then cooled. Granulated zinc is added prior to the addition of tap water and an excess of 40% NaOH. The zinc prevents bumping and frothing when the NaOH is added whereas the NaOH neutralizes the concentrated acid and releases the ammonia from its fixed state of ammonium sulfate. Potassium sulfide present in the NaOH solution reacts with the Hg in the catalyst producing HgS, thus preventing the Hg from distilling over and interfering with the end point. The ammonia is then immediately steam distilled into boric acid and the ammonia-boric acid complex is then acid titrated with standard acid.

d) Analysis of data

Individual values, means, and standard error of the mean are presented for each index measured. Doubtful observations were rejected according to the procedure described by Fales and Kenny (40). To test the significance of differences between the values of the control group and the experimental group the unpaired t-test (41) was used. A paired t-test (42) was used to determine the levels of significance of the differences in the means of indices in the same group before and after the tryptophan load dose.

---

[1]Matheson Scientific, East Rutherford, New Jersey.

RESULTS AND DISCUSSION

Pre-Study Analyses

a) Pre-study clinical examination

The pre-study clinical examination showed all subjects to be in good health. Results of the laboratory investigations conducted are shown in Table 6.

The hemoglobin level was determined using a cyanmethemoglobin method. All participants had hemoglobin levels within the range of a healthy adult woman (35). The unpaired t-test showed no significant difference between the control and experimental groups.

Serum glutamic oxalacetic transaminase (SGOT) levels were used as a test of liver function for, in the presence of hepatitis, serum enzyme activity is increased presumably due to liberation of SGOT from injured cells (43). Larsson-Cohn (44) previously reported elevated SGOT levels in 6% of a group of 284 women aged 18 to 46 years who had received an oral contraceptive preparation consisting of 2 mg of norethindrone (progestogen) and 0.075 mg mestranol (estrogen) daily for one year. The SGOT levels of all subjects in this study were within the normal range of 5 to 40 units with the exception of subject 8 who had a slightly elevated SGOT level of 46 units. No significant difference between the control group and the experimental group was found using the unpaired t-test.

Whether or not urinary glucose was present was determined using Hema-Combistix.[1] Several reports have indicated a possibility of

---

[1] Ames Company, Division Miles Laboratory Ltd., Rexdale, Ontario.

Table 6

Pre-study clinical examination laboratory results

Subject	Hemoglobin level[1]	SGOT level[2,3]	Urinary Glucose[4]	Urinary Albumin[4]
Control Group				
1	13.7	24	-	-
2	13.9	33	-	-
3	14.8	27	-	-
4	14.7	25	-	-
5	13.7	19	-	-
Mean value	14.2	26		
SEM[5]	±0.2	±2		
Experimental Group				
6	12.9	20	-	-
7	14.5	28	-	-
8	13.0	46	-	-
9	13.0	35	-	-
10	13.3	27	-	-
Mean value	13.3	31		
SEM[5]	±0.3	±4		

1 Expressed as gm/100 ml blood.

2 Serum glutamic oxalacetic transaminase.

3 Expressed as units.

4 - Indicates negative.

5 Standard error of the mean.

impaired glucose tolerance in those taking oral contraceptives (45-47). In this study no glucose was detected in the urine of any of the participants.

The presence of albumin in the urine, indicative of renal damage, was determined using sulfosalicylic acid. No albumin was found in the urine of any of the subjects.

b) Pre-study diet record

The three-day pre-study diet record (Table 7) showed considerable individual variations in food consumption, however, there was no significant difference in intake of calories, protein or vitamin B_6 between the control and experimental group using the unpaired t-test.

Study Analyses

a) Ad libitum consumption

The average daily ad libitum caloric consumption, the resulting total daily caloric intake, and the caloric intake/pound of body weight (Table 8) showed no significant difference between the control and experimental group as determined using the unpaired t-test.

b) Basal body temperature record

The basal body temperature record kept by the control group from the onset of the menstrual period to day 20 of the menstrual cycle showed a diphasic character (Figure 4). In all 5 subjects the upward shift in basal body temperature indicating ovulation, occurred on or very near day 14 of the cycle. The calculated ovulation time thus coincided with the actual ovulation time in these 5 subjects.

Table 7

Pre-study diet record. Average daily calculated consumption

Subject	Kcal.[1,2]	Protein[2,3]	Vitamin B_6[4,5]
Control Group			
1	1288	57.1	1.042
2	1170	81.6	1.119
3	1493	61.8	1.119
4	2286	98.8	1.783
5	1565	51.4	0.754
Mean value	1560	70.1	1.163
SEM[6]	±195	±8.8	±0.169
Experimental Group			
6	2101	68.8	1.127
7	1586	51.7	1.149
8	1508	80.4	1.599
9	1551	65.3	0.960
10	2617	93.8	1.314
Mean value	1873	72.0	1.230
SEM[6]	±215	±7.1	±0.108

1 Kilocalories.

2 Calculated from the figures of Watt and Merrill (48) and Bogert et al. (49).

3 Expressed as gm.

4 Expressed as mg.

5 Calculated from the figures of Polansky (50), Polansky and Murphy (51), Polansky et al. (52), Hardinge and Crooks (53), Lieck and Søndergaard (54), Toepfer et al. (55), and Orr (56).

6 Standard error of the mean.

Table 8

Average daily ad libitum consumption, total daily caloric intake, and caloric intake/pound of body weight

Subject	Ad libitum consumption[1]	Total caloric intake [1,2]	Kcal[1]/lb body weight
Control Group			
1	0	1378	12
2	264	1642	13
3	74	1452	12
4	160	1538	13
5	360	1738	13
Mean value	172	1550	13
SEM[3]	±65	±65	±0.2
Experimental Group			
6	241	1619	10
7	712	2090	17
8	99	1477	12
9	61	1439	13
10	406	1784	14
Mean value	304	1682	13
SEM[3]	±119	±119	±1.2

1 Expressed as kilocalories.

2 Total caloric intake = basal caloric intake + ad libitum where basal caloric intake = 1378 kilocalories.

3 Standard error of the mean.

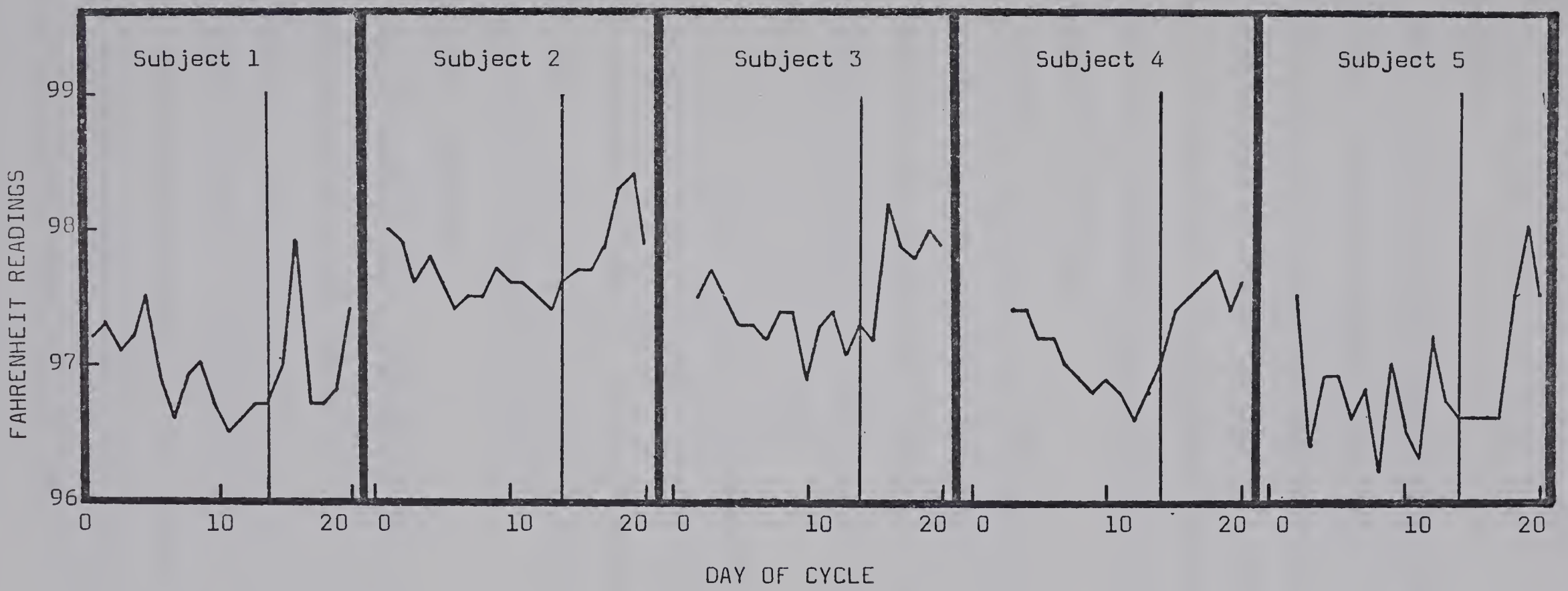

Figure 4 Control group basal temperature record.

c) Creatinine

Daily creatinine values showed slight variations both within the individual and between individuals (Table 9). The day to day variations within the individual suggests that it is questionable as to how rigidly such values can be used as a definite check for completeness of collection. No significant difference existed between the control and experimental group using the unpaired t-test.

d) Hemoglobin and hematocrit

Hemoglobin and hematocrit levels as determined on day 6 of the study (Table 10) are in agreement with those of previous studies (57, 58). The difference between the control and experimental group for both hemoglobin and hematocrit values was not significant as indicated by analysis using the unpaired t-test.

For some subjects there was considerable difference in hemoglobin values determined on day 6 compared with those reported in the pre-study clinical examination (Table 6). This may be due to the time lapse of several weeks in some instances between the two analyses or perhaps due to the different methods of analysis.

e) Tryptophan metabolites

i) Xanthurenic acid. Xanthurenic acid excretion before and after consumption of a 2-gm load dose of L-tryptophan appears in Table 11. There was no significant difference in xanthurenic acid excretion in the days 1-4 composite compared with day 5 in either the control group or the experimental group using a paired t-test; however, following the tryptophan loading on day 6 the control group excreted a significantly greater amount of xanthurenic acid ($P < 0.001$) than before

Table 9

Daily creatinine[1] production

Subject	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Mean	SEM[2]
Control Group								
1	0.7	0.8	0.7	1.0	1.2*	0.9	0.9	±0.1
2	1.0	1.1	1.1	1.1	1.0	1.1	1.1	±0.0
3	1.0	1.1	1.2	1.2	1.2	1.2	1.2	±0.0
4	1.0	1.0	1.0	1.0	1.0	1.1	1.0	±0.0
5	1.2	1.1	1.2	1.2	1.2	1.2	1.2	±0.0
Group Mean							1.1	
Experimental Group								
6	1.3	1.4	1.0	1.6	1.5	1.3	1.4	±0.1
7	1.1	0.9	1.0	0.9	1.0	0.9	1.0	±0.0
8	1.1	1.2	0.9	1.1	1.1	1.1	1.1	±0.0
9	1.1	1.0	1.1	1.1	1.0	1.1	1.1	±0.0
10	1.3	1.2	1.2	1.2	1.2	1.2	1.2	±0.0
Group Mean							1.2	

1 Values are expressed as gm creatinine/24 hours.

2 Standard error of the mean.

* Approximately 100 ml of day 6 urine sample was placed in day 5 composite.

Table 10

Hemoglobin and hematocrit levels as determined on day 6 of the study

Subject	Hemoglobin[1]	Hematocrit[2]
Control Group		
1	12.7	36
2	15.0	40
3	15.9	44
4	15.0	42
5	14.6	43
Mean	14.6	41
SEM[2]	±0.5	±1
Experimental Group		
6	14.6	40
7	14.6	39
8	13.9	38
9	12.7	36
10	14.2	39
Mean	14.0	38
SEM[2]	±0.3	±1

1 Values are expressed as gm/100 ml of blood.

2 Percentage packed cells.

3 Standard error of the mean.

Table 11

Xanthurenic acid[1] excretion in the urine of 5 women using oral contraceptives and in 5 controls before and after taking a load dose of 2 gm of L-tryptophan

Subject	Pre-load		Post-load
	Days 1-4	Day 5	Day 6
Control Group (C)			
1	10.4	12.2*	59.9
2	12.0	10.7	56.8
3	11.3	11.3	46.5
4	10.3	7.8	50.4
5	14.0	12.4	51.6
Mean	11.6	10.9	53.0
SEM[2]	±0.7	±0.8	±2.4
Level of Significance	N.S.[3]	P < 0.001	
Experimental Group (E)			
6	15.6	18.2	461.3
7	15.4	15.8	441.4
8	7.5	10.0	168.8
9	17.2	15.0	430.1
10	24.8	22.3	729.9
Mean	16.1	16.3	446.3
SEM[2]	±2.8	±1.9	±88.9
Level of Significance	N.S.[3]	P < 0.01	
Level of Significance: E versus C	N.S.[3]	P < 0.05	P < 0.01

1 Values expressed as μmoles/24 hours.

2 Standard error of the mean.

3 Not significant.

* Approximately 100 ml of day 6 urine sample was placed in day 5 composite.

loading as did the experimental group ($P<0.01$). Comparison of xanthurenic acid excretion in the control group versus the experimental group using an unpaired t-test showed no significant difference on the days 1-4 composite but a significantly greater excretion by the experimental group on day 5 ($P<0.05$) and day 6 ($P<0.01$).

Subject 8, although taking a preparation similar in dosage to the other subjects (Table 4), excreted consistently lower levels of xanthurenic acid than did the other experimental subjects. According to the pre-study diet record (Table 7) she ingested a relatively high daily amount of vitamin B_6 prior to the study which could suggest that she had a larger reserve of vitamin B_6 than did the other subjects which might explain the lack of response to the load dose. However, this result could also be due to an unexplained individual metabolic alteration.

The trend of an increased xanthurenic acid excretion in the users of oral contraceptives in this study agreed with the previous work of Price et al. (17) and Aly et al. (18). Price et al. (Table 2), however, reported a lower mean level of xanthurenic excretion by the control group following loading than is reported in this study and a higher post-tryptophan xanthurenic acid excretion by the experimental group than reported here. In comparison, Aly et al. (Table 3) found a greater mean xanthurenic acid excretion in both groups prior to the tryptophan load and a lesser amount of xanthurenic acid excreted by the experimental group following loading than in the present study.

ii) Kynurenic acid. In the present study there was no significant difference in kynurenic acid excretion (Table 12) in either the

Table 12

Kynurenic acid[1] excretion in the urine of 5 women using oral contraceptives and in 5 controls before and after taking a load dose of 2 gm of L-tryptophan

Subject	Pre-load		Post-load
	Days 1-4	Day 5	Day 6
Control Group (C)			
1	23.2	25.8*	156.1
2	15.8	14.2	89.2
3	16.9	16.2	90.0
4	11.3	8.1	49.3
5	14.5	13.0	88.0
Mean	16.3	15.5	94.5
SEM[2]	±2.0	±2.9	±17.2
Level of Significance	N.S.[3]	P< 0.01	
Experimental Group (E)			
6	11.8	11.2	121.8
7	11.2	12.0	142.9
8	3.8	6.5	53.4
9	8.6	6.4	102.5
10	7.9	6.5	85.6
Mean	8.7	8.5	101.2
SEM[2]	±1.4	±1.3	±15.5
Level of Significance	N.S.[3]	P< 0.01	
Level of Significance: E versus C	P< 0.02	N.S.[3]	N.S.[3]

1 Values expressed as μmoles/24 hours.

2 Standard error of the mean.

3 Not significant.

* Approximately 100 ml of day 6 urine sample was placed in day 5 composite.

experimental or the control group when the days 1-4 composite was compared with day 5 using a paired t-test. Following loading, however, a significantly greater amount of kynurenic acid was excreted compared with pre-load values in both the control group ($P < 0.01$) and the experimental group ($P < 0.01$). Comparison of the control group with the experimental group using the unpaired t-test indicated that the control group excreted a significantly greater ($P < 0.02$) amount of kynurenic acid in the days 1-4 composite whereas no significant difference was noted for day 5 or day 6.

Subject 1 excreted higher levels of kynurenic acid than did the other control subjects. This could be due to a prolonged intake of a lower level of vitamin B_6 although this was not indicated in the pre-study diet record (Table 7). Following tryptophan loading, Subject 8 produced lower amounts of kynurenic acid than did the rest of the experimental group. This same subject also had a decreased excretion of xanthurenic acid when compared with other members of her group.

In contrast to the present study, Price et al. (17) had noted a significantly greater excretion of kynurenic acid following loading by the users of oral contraceptives compared to those not using the pill (Table 2). The mean post-tryptophan kynurenic acid level of the experimental group in the present study was very near that reported by Price et al., however, the mean level of kynurenic acid excreted by the control group following the load dose was greater in the present study than in the study of Price et al. (17).

Aly et al. (18) reported a higher mean pre-tryptophan value for the experimental group (Table 3) and much lower post-tryptophan values

for both groups. In agreement with the present study Aly et al. did not find a significant difference in kynurenic acid excretion between the experimental and control group following the tryptophan load dose.

iii) N^1-methylnicotinamide. N^1-methylnicotinamide excretion was measured before and after administration of a 2-gm oral dose of L-tryptophan (Table 13). There was no significant mean difference in pre-load excretion of N^1-methylnicotinamide in either the control group or the experimental group when the days 1-4 composite was compared with day 5 using a paired t-test. However, following loading a significantly greater mean amount of N^1-methylnicotinamide was produced in both the control group ($P<0.05$) and the experimental group ($P<0.02$) when compared with pre-load values using a paired t-test. Comparison of the control group with the experimental group using an unpaired t-test showed a significantly greater ($P<0.05$) mean production of N^1-methylnicotinamide by the experimental group in the days 1-4 composite, day 5 and day 6.

Subject 8, although an experimental subject, produced values for N^1-methylnicotinamide which were similar to those of the control group.

Rose et al. (19) also found a statistically significantly greater mean excretion of N^1-methylnicotinamide in the experimental group compared with the control group both before and after a 2-gm load dose of L-tryptophan. Rose et al. however reported mean excretion levels of 32 μmoles/24 hours in the control group and 60 μmoles/24 hours in the experimental group which were lower than those of the present study. Following a 2-gm load dose, Rose et al. reported a greater excretion by the control group (75 μmoles/24 hours) and lesser

Table 13

N^1-methylnicotinamide[1] excretion in the urine of 5 women using oral contraceptives and in 5 controls before and after taking an oral dose of 2 gm of L-tryptophan

Subject	Pre-load		Post-load
	Days 1-4	Day 5	Day 6
Control Group (C)			
1	46.5	61.3*	59.8
2	60.5	49.0	75.2
3	53.6	52.4	68.0
4	70.7	53.4	75.6
5	59.6	61.0	64.8
Mean	58.2	55.4	68.7
SEM[2]	±4.0	±2.4	±3.0
Level of Significance	N.S.[3]	P<0.05	
Experimental Group (E)			
6	130.6	161.2	232.9
7	66.6	79.1	123.1
8	52.5	56.9	79.7
9	125.6	166.4	283.2
10	116.5	116.1	135.9
Mean	98.4	115.9	171.0
SEM[2]	±16.2	±21.7	±37.6
Level of Significance	N.S.	P<0.02	
Level of Significance: E versus C	P<0.05	P<0.05	P<0.05

1 Values expressed as μmoles/24 hours.

2 Standard error of the mean.

3 Not significant.

* Approximately 100 ml of day 6 urine sample was placed in day 5 composite.

production by the experimental group (148 μmoles/24 hours) than in the present study.

Although the experimental subjects had taken oral contraceptives of varying estrogen-progestogen composition and had been using the pills for varying lengths of time (Table 4), these differences were not reflected in the relative amounts of tryptophan metabolites excreted. Table 14 summarizes the urinary yield of the tryptophan metabolites measured in the present study compared with the values obtained in other studies (17-19).

Several theories have been proposed to explain the abnormal tryptophan metabolism which exists in those using oral contraceptives, but as yet no single theory has entirely explained the problem. With respect to the present study the following proposals must be considered:

aa) An increased tryptophan pyrrolase activity (fig.1) as a direct result of the use of estrogenic steroids could result in an increased production of tryptophan metabolites all along the tryptophan to pyridone pathway and, therefore, an increased requirement for vitamin B_6. The increase of xanthurenic acid and N^1-methylnicotinamide in the present study would support this hypothesis. This theory is based upon the hypothesis proposed by Rose (59) that tryptophan pyrrolase activity is mediated by way of the hypothalamo-pituitary-adrenal axis. The administration of estrogens stimulates this axis to produce increased levels of plasma 17-hydroxycorticosteroids (60) which in turn cause the induction of the enzyme tryptophan pyrrolase (61).

This theory, however, fails to explain why pyridone (fig.1) levels are not also increased in those taking oral contraceptives

Table 14

Comparison of mean urinary yield[1] of tryptophan metabolites following a 2-gm load dose of L-tryptophan

	XA[2]		KA[3]		N'MN[4]	
	Control	OCA[5]	Control	OCA[5]	Control	OCA[5]
Present study	42	430*	79	93	14	55*
Price et al. (17)	22	686*	46	90*	--	--
Aly et al. (18)	58	263*	39	42	--	--
Rose et al. (19)	--	--	--	--	44	88*

1 Yield = (post-tryptophan) - basal excretion.

2 Xanthurenic acid excretion expressed as μmoles/24 hours.

3 Kynurenic acid excretion expressed as μmoles/24 hours.

4 N'-methylnicotinamide excretion expressed as μmoles/24 hours.

5 Subjects taking oral contraceptive agents.

* Indicates values which differ significantly from the control value according to the Student's t-test at a $P < 0.05$.

(17,19) and also why with pyridoxine administration to oral contraceptive users tryptophan metabolite levels are decreased (16,17) and not increased as would be expected if induction of tryptophan pyrrolase has occurred. It also fails to explain in the present study why kynurenic acid levels were not significantly elevated in the experimental group.

bb) There could be a complete or partial blockage in the enzymatic pathway occurring after the formation of 3-hydroxykynurenine because kynurenine transaminase and kynureninase (fig.1) are vitamin B_6 dependent enzymes and in oral contraceptive users a relative shortage of vitamin B_6 could occur due to the increased need of this group for vitamin B_6. Other workers have also reported possible hormonal inhibition of these 2 enzymes (62). The transaminase enzymes have a greater affinity for vitamin B_6 than kynureninase which results in increased production of xanthurenic and kynurenic acid (11). The formation of kynurenic acid is thought to be more sensitive to vitamin B_6 depletion thus explaining why xanthurenic acid accumulates to a greater extent than kynurenic acid. This occurred in the present study. Supplementation with vitamin B_6 has been reported to correct the abnormally high amounts of xanthurenic and kynurenic acid excreted in oral contraceptive users (16, 17) thus giving validity to this theory.

This explanation fails, however, to explain the increased production of N^1-methylnicotinamide by the experimental group as reported here and by Rose et al. (19). They (19) suggest that this could be a result of a combination of enhanced tryptophan pyrrolase activity accompanied by N^1-methylnicotinamide oxidase inhibition by estrogens.

cc) A third theory could be that there is a redistribution of vitamin B_6 within the body tissues due to induced synthesis of other pyridoxine-requiring enzymes thus resulting in a vitamin B_6 deficiency with respect to the tryptophan-niacin pathway in those taking oral contraceptives. This has been suggested by Brin (63) who noted estrogenic induction in the rat liver of the vitamin B_6-dependent enzyme pyruvate amino-transferase.

A combination of the above hypotheses may explain the cause of the abnormal tryptophan metabolism resulting in those taking oral contraceptives. It is possible that several blockages occur in the tryptophan-niacin pathway each of which could have a different remedy. Most authors agree, however, that there seems to be an increased need for vitamin B_6 in those taking oral contraceptives. How large this requirement is and whether or not large doses of vitamin B_6 will totally alleviate the abnormal tryptophan metabolism in this group awaits further study. The need for vitamin B_6 so far has been based only on the correction of the abnormality of the tryptophan-niacin pathway. Perhaps the involvement of vitamin B_6 in other areas, such as the formation of serotonin or as the coenzyme for glutamic oxaloacetic transaminase and glutamic pyruvate transaminase, should be considered when establishing the requirement for vitamin B_6 in oral contraceptive users.

f) Nitrogen

A summary of the data obtained in the present study from a comparison of urinary nitrogen excretion in the control and experimental groups before and after tryptophan loading appears in Table 15. No

Table 15

Nitrogen[1] excretion in the urine of 5 women using oral contraceptives and 5 controls before and after taking an oral dose of 2 gm L-tryptophan

Subject	Pre-load Days 1-4	Pre-load Day 5	Post-load Day 6
Control Group (C)			
1	10.4	11.4*	10.6
2	10.6	9.9	10.6
3	10.5	11.2	11.1
4	10.3	10.7	10.1
5	11.0	7.2	9.9
Mean	10.6	10.1	10.5
SEM[2]	±0.1	±0.8	±0.2
Level of Significance	N.S.[3]		N.S.[3]
Experimental Group (E)			
6	8.7	10.7	11.2
7	9.9	10.1	8.3
8	9.5	10.0	11.4
9	10.7	10.9	11.0
10	11.6	11.9	11.6
Mean	10.1	10.7	10.7
SEM[2]	±0.5	±0.3	±0.6
Level of Significance	N.S.		N.S.
Level of Significance: E versus C	N.S.	N.S.	N.S.

1 Values expressed as gm/24 hours.

2 Standard error of the mean.

3 Not significant.

* Approximately 100 ml of day 6 urine sample was placed in day 5 composite.

significant differences were noted either when pre-load values were compared with post-load values (paired t-test) or when the experimental group was compared with the control group (unpaired t-test).

These results confirm that there is no increase in urinary nitrogen loss in oral contraceptive users. This agrees with results obtained by Craft et al. (64) who found no significant increase in urinary amino acid or total nitrogen in those taking oral contraceptives. They also reported a fall in plasma amino acids occurring early in the menstrual cycle soon after starting the course of oral contraceptive tablets and persisting until after the completion of the monthly tablet routine. Other workers (18, 21, 22) had also found decreased plasma amino acid levels in subjects taking oral contraceptives.

There is little evidence that estrogens have any marked effect on free plasma and urinary acids in man (23) and therefore the progestogenic component is more likely responsible for this effect.

In possible explanation as to the location of the shift of amino acids from the plasma in those taking oral contraceptives Lecocq et al. (65) described an increase in lean body mass due to protein accumulation in young women maintained on an estrogen preparation to which a progestogen was added (either 5 mg of norethynodrel daily or 2 mg chlormadinone daily). They suggest that these findings were due to the anabolic effects of progesterone on peripheral tissue and probably resulted from increased tissue utilization of amino acids. This may also help to explain the tendency to gain weight experienced by some women taking oral contraceptives. Further studies are indicated

to determine the extent of this increased tissue utilization of amino acids in oral contraceptive users.

SUMMARY

1. The pre-study clinical examination showed all subjects to be in good health. No significant differences in hemoglobin or serum glutamic oxalacetic transaminase levels were noted when the control and experimental groups were compared. No urinary glucose or albumin was detected in any of the participants.

2. The 3-day pre-study diet record showed no significant difference in intake of calories, protein, or vitamin B_6 between the control and experimental group.

3. The average daily ad libitum caloric consumption and the resulting caloric intake per pound of body weight for the control group was not significantly different from the experimental group.

4. In all 5 control subjects the upward shift in basal body temperature, indicating ovulation, occurred on or very near day 14 of the cycle.

5. There were slight variations in daily creatinine levels both within the individual and between individuals, however, no significant difference existed between the control and experimental group.

6. The difference in both hemoglobin and hematocrit levels as determined on day 6 of the study was not significant when the control group was compared with the experimental group.

7. The levels of the urinary tryptophan metabolites: xanthurenic acid, kynurenic acid and N^1-methylnicotinamide, were not significantly different for either the control group or the experimental group when the days 1-4 composite was compared with the day 5 sample; however

following the 2-gm load dose of L-tryptophan a significant increase was noted in both the control and experimental groups for all tryptophan metabolites measured.

8. Comparison of the control group with the experimental group showed no significant difference in xanthurenic acid excretion for the composite made for days 1-4, whereas the experimental group excreted significantly greater amounts of xanthurenic acid than did the control group on day 5 and day 6.

9. Urinary kynurenic acid excretion was significantly increased in the control group compared with the experimental group for the days 1-4 composite, however, there was no significant difference between experimental and control groups for day 5 or day 6.

10. The excretion of N^1-methylnicotinamide in the days 1-4 composite or on day 5 was not significantly different for the experimental group compared to the control group, but a significantly greater excretion by the experimental group when compared to the control group occurred on day 6.

11. No significant differences in nitrogen excretion were noted either when pre-tryptophan load and post-tryptophan load values were compared or when the control group was compared with the experimental group.

BIBLIOGRAPHY

1. Drill, U. A. 1970 Antifertility drugs. Introductory remarks. Federation Proc., 29: 1209.

2. National Research Council 1968 Recommended Dietary Allowances, rev. ed. 7. National Academy of Sciences, Washington, D.C., publ. no. 1694.

3. Donald, E. A., L. D. McBean, M. H. W. Simpson, M. F. Sun and H. E. Aly 1971 Requirement for vitamin B_6 by young adult women. Amer. J. Clin. Nutr., 24: 1028.

4. Luhby, A. L., M. Brin, M. Gordon, P. Davis, M. Murphy, and H. Spiegel 1971 Vitamin B_6 metabolism in users of oral contraceptive agents. I. Abnormal urinary xanthurenic acid excretion and its correction by pyridoxine. Amer. J. Clin. Nutr., 24: 684.

5. Saunders, F. J. 1970 Endocrine properties and mechanism of action of oral contraceptives. Federation Proc., 29: 1211.

6. Osbond, J. M. 1964 Synthesis and labeling of the vitamin B_6 group. In: Vitamins and Hormones, eds., R. S. Harris, I. G. Wool and J. A. Loraine. Academic Press, New York, 22: 367.

7. Snell, E. E. 1958 Chemical structure in relation to biological activities of Vitamin B_6. In: Vitamins and Hormones, eds., R. S. Harris, G. F. Marrian and K. V. Thimann. Academic Press, New York, 16: 77.

8. Brown, R. R., D. P. Rose, J. M. Price and H. Wolf 1969 Tryptophan metabolism as affected by anovulatory agents. Ann. N.Y. Acad. Sci., 166: 44.

9. Yess, N., J. M. Price, R. R. Brown, P. B. Swan and H. Linkswiler 1964 Vitamin B_6 depletion in man: urinary excretion of tryptophan metabolites. J. Nutr., 84: 229.

10. Coursin, D. B. 1964 Recommendations for standardization of the tryptophan load test. Am. J. Clin. Nutr., 14:56.

11. Ogasawara, N., Y. Hagino and Y. Kotake 1962 Kynurenine-transaminase, kynureninase and the increase of xanthurenic acid excretion. J. Biochem. (Tokyo), 52: 162.

12. Greenberg, L. D., D. F. Bohr, H. McGrath and J. F. Rinehart 1949 Xanthurenic acid excretion in the human subject on a pyridoxine-deficient diet. Arch. Biochem., 21: 237.

13. Price, J. M., R. R. Brown, and N. Yess 1965 Testing the functional capacity of the tryptophan-niacin pathway in man by analysis of urinary metabolites. In: Advances in Metabolic Disorders. eds. R. Levine and R. Luft. Academic Press, New York, 2: 159.

14. Price, J. M., R. R. Brown and F. C. Larson 1957 Quantitative studies on human urinary metabolites as affected by isoniazid and deoxypyridoxine. J. Clin. Invest., 36: 1600.

15. Rose, D. P. 1966 Excretion of xanthurenic acid in the urine of women taking progestogen-oestrogen preparations. Nature, 210: 196.

16. Rose, D. P. 1966 The influence of oestrogen on tryptophan metabolism in man. Clin. Sci., 31: 265.

17. Price, J. M., M. J. Thornton and L. M. Mueller 1967 Tryptophan metabolism in women using steroid hormones for ovulation control. Amer. J. Clin. Nutr., 20: 452.

18. Aly, H. E., E. A. Donald and M. W. Simpson 1971 Oral contraceptives and vitamin B_6 metabolism. Amer. J. Clin. Nutr., 24: 297.

19. Rose, D. P., R. R. Brown and J. M. Price 1968 Metabolism of tryptophan to nicotinic acid derivatives by women taking oestrogen-progestogen preparations. Nature, 219: 1259.

20. Glüecksohn-Waelsch, S., P. Greengard, G. P. Quinn and L. S. Teicher 1967 Genetic variations of an oxidase in mammals. J. Biol. Chem., 242: 1271.

21. Craft, I. L. and I. Wise 1969 Oral contraceptives and plasma amino acids. Nature, 222: 487.

22. Friedberg, F. and D. M. Greenberg 1947 Endocrine regulation of amino acid levels in blood and tissues. J. Biol. Chem., 168: 405.

23. Zinneman, H. H., B. U. Musa and R. P. Doe 1965 Changes in plasma and urinary amino acids following estrogen administration to males. Metabolism, 14: 1214.

24. Zinneman, H. H., U. S. Seal and R. P. Doe 1967 Urinary amino acids in pregnancy, following progesterone, and estrogen-progestogen. J. Clin. Endocrinol. and Metab., 27: 397.

25. McCorquodale, D. J. and G. C. Mueller 1958 Effect of estradiol on the level of amino acid-activating enzymes in the rat uterus. J. Biol. Chem., 232: 31.

26. Tritze, C. 1968 Oral and intrauterine contraception, effectiveness and safety. Int. J. Fert., 13: 377.

27. Department of National Health and Welfare, Canada 1963 Canadian Bulletin on Nutrition. Dietary Standard for Canada. Department of Public Printing and Stationery, Ottawa, Canada, vol. 6., no. 1.

28. Rose, D. P. 1967 The influence of sex, age, and breast cancer on tryptophan metabolism. Clin. Chim. Acta., 18: 221.

29. Palmer, A. 1953 The diagnostic use of the basal body temperature in gynecology and obstetrics. In: Gynecology and Obstetrics, ed., C. H. Davis. p.57.

30. Horrobin, D. F. 1968 Medical Physiology and Biochemistry, Edward Arnold Ltd., London, p.138.

31. Oser, B. L. 1965 Hawk's Practical Physiological Chemistry. 14th edition. McGraw-Hill, New York, p.1233.

32. Bausch and Lomb 1965 Creatinine in serum or urine. In: Spectronic 20 Clinical Methods Manual, Bausch and Lomb Inc., Rochester, p.31.

33. Rapoport, M. I. 1968 Circadian periodicity of tryptophan metabolism. J. Clin. Invest., 47: 934.

34. Feign, R. D., A. S. Klainer and W. R. Beisel 1968 Factors affecting circadian periodicity of blood amino acids in man. Metabolism, 17: 764.

35. Bausch and Lomb 1965 Hemoglobin (as oxyhemoglobin) in blood. In: Spectronic 20 Clinical Methods Manual, Bausch and Lomb Inc., Rochester, p.41.

36. Price, J. M. and L. W. Dodge 1956 Occurrence of the 8-methyl ether of xanthurenic acid in normal human urine. J. Biol. Chem., 223: 699.

37. Pelletier, O. and J. A. Campbell 1962 A rapid method for the determination of N^1-methylnicotinamide in urine. Anal. Biochem., 3: 60.

38. Huff, J. W. 1947 The fluorescent condensation product of N^1-methylnicotinamide and acetone. I. Synthesis and properties. J. Biol. Chem., 167: 151.

39. Association of Official Agricultural Chemists 1960 Official Methods of Analysis of the Association of Official Agricultural Chemists, 9th edition, Washington, D.C., p.12.

40. Fales, H. A. and F. Kenny 1939 Inorganic Quantitative Analysis, D. Appleton-Century Co., New York, p.77.

41. Steel, R. G. D. and J. H. Torrie 1960 Principles and Procedures of Statistics. McGraw-Hill, New York, p.73.

42. Steel, R. G. D. and J. H. Torrie 1960 Principles and Procedures of Statistics. McGraw-Hill, New York, p.78.

43. Merck Manual 1966 11th edition, Merck and Co. Inc., Rathway, New Jersey, p.916.

44. Larsson-Cohn, U. 1965 Oral contraceptives and liver function tests. Brit. Med. J., 1: 1414.

45. Gershberg, H., Z. Javier and M. Hulse 1964 Glucose tolerance in women receiving an ovulatory suppressant. Diabetes, 13: 378.

46. Wynn, V. and J. W. H. Doar 1966 Some effects of oral contraceptives on carbohydrate metabolism. Lancet, 2: 715.

47. Buckler, D. and J. C. Warren 1966 Effects of estrogen on glucose tolerance. Amer. J. Obstet. Gynec., 95: 479.

48. Watt, B. K. and A. L. Merrill 1963 Composition of Foods, Agriculture Handbook No. 8, U.S. Dept. of Agric., Washington, D.C.

49. Bogert, L. J., G. M. Brigg and D. H. Callaway 1966 Nutrition and Physical Fitness, 8th ed. W. B. Saunders Co., Philadelphia, pp.558-579.

50. Polansky, M. M. 1969 Vitamin B_6 components in fresh and dried vegetables. J.A.D.A., 54: 118.

51. Polansky, M. M. and E. W. Murphy 1966 Vitamin B_6 components in fruits and nuts. J.A.D.A., 48: 109.

52. Polansky, M. M., E. W. Murphy, and E. W. Toepfer 1964 Components of vitamin B_6 in grains and cereal products. J.A.O.A.C., 47: 750.

53. Hardinge, M. G. and H. Crooks 1961 Lesser known vitamins in foods. J.A.D.A., 38: 240.

54. Lieck, H. and H. Søndergaard 1958 The content of vitamin B_6 in Danish foods. Intl. Rev. Vitamin Res. (Bern), 29: 68.

55. Toepfer, E. W., M. M. Polansky, L. R. Richardson and S. Wilkes 1963 Comparison of vitamin B_6 values of selected food samples by bioassay and microbiological assay. J. Agr. and Food Chem., 11: 523.

56. Orr, M. L. 1969 Pantothenic Acid, Vitamin B_6 and Vitamin B_{12} in Foods. Home Economics Research Report No. 36, U.S. Dept. of Agriculture.

57. Robertson, G. S. 1967 Serum protein and cholinesterase changes in association with contraceptive pills. Lancet, 1: 232.

58. Burton, J. L. 1967 Effect of oral contraceptives on hemoglobin, packed-cell volume, serum-iron, and total iron-binding capacity in healthy women. Lancet, 1: 978.

59. Rose, D. P. 1969 Oral contraceptives and depression. Lancet, 2: 321.

60. Wallace, E. Z., H. I. Silverberg and A. C. Carter 1957 Effect of ethinyl estradiol on plasma 17-hydroxycorticosteroids, ACTH responsiveness and hydrocortisone clearance in man. Proc. Soc. Exptl. Biol. Med., 95: 805.

61. Rose, D. P. and I. P. Braidman 1971 Excretion of tryptophan metabolites as affected by pregnancy, contraceptive steroids, and steroid hormones. Am. J. Clin. Nutr., 24: 673.

62. Mason, M. and B. Manning 1971 Effects of steroid conjugates on availability of pyridoxal phosphate for kynureninase and kynurenine aminotransferase activity. Am. J. Clin. Nutr., 24: 786.

63. Brin, M. 1971 Abnormal tryptophan metabolism in pregnancy and with the oral contraceptive pill. I. Specific effects of an oral estrogenic contraceptive steroid on the tryptophan oxygenase and two aminotransferase activities in livers of ovarectomized-adrenalectomized rats. Am. J. Clin. Nutr. 24: 699.

64. Craft, I. L., I. J. Wise and M. H. Briggs 1970 Oral contraceptives and amino acid utilization. Amer. J. Obstet. Gynec., 108: 1120.

65. Lecocq, F. R., E. M. Bradley and J. W. Goldzieher 1967 Metabolic balance studies with norethynodrel and chlormadinone acetate. Amer. J. Obstet. Gynec., 99: 374.

APPENDICES

Appendix I

Composition of the Diet

A. Basal diet (Consumed by each subject daily)

Carnation Instant Breakfast: 4 envelopes each weighing 1.33 oz (choice of vanilla, chocolate, chocolate malt, chocolate marshmallow, coffee, eggnog or strawberry flavor)

Powdered skim milk: 80 gm

Saltine crackers: 6 crackers each weighing 12 gm

Processed Canadian cheese: 2 oz

Canned apple sauce: 8 fl oz

The basal diet was calculated to contain 81 gm protein, 1378 calories and 1.3 mg of Vitamin B_6 as previously reported by Aly et al. (18).

B. Ad libitum diet

Butter

White granulated sugar

Candy (buttermints and/or creams)

Shortbread cookies

Instant coffee and/or instant tea (limited to 1.5 gm of coffee per cup or 0.4 gm of tea per cup to a maximum of 4 cups total beverage intake per day).

Appendix II

Urine Compositing and Storage

A. Following each 24-hour collection:

1. Composite the individual bottles in a 2000 ml graduated cylinder. Record the daily volume.

2. Bring the urine volume to the nearest 100 ml with distilled water. Record volume.

3. Set aside an approximate 10 ml portion of urine in a labelled 4 oz bottle for the determination of creatinine. Label, cap, and freeze at -20°C.

4. Fill one 16 oz bottle 1/2 to 2/3 full with urine. Label, cap and store at -20°C.

B. Days 1-4:

Place 25% of the volume of urine excreted for each of day 1 through day 4 in a labelled 2 litre bottle in the refrigerator.

C. For each of days 1-4 composite, day 5 and day 6:

1. Fill one 16 oz bottle 1/2 to 2/3 full with urine for kynurenic acid and xanthurenic acid determinations. Label, cap, and store at -20°C.

2. Fill approximately 2/3 full with urine two 4 oz bottles for N^1-methylnicotinamide and nitrogen determinations. Label, cap and store at -20°C.

3. Acidify to pH 1 to 1.5 approximately 30 to 40 ml of urine using several drops of 6 N HCl. Test with hydrion paper. Spin at

15,000 rpm for 15 minutes on the Sorvall Superspeed SS-3 centrifuge to remove any sediment. If supernatant is cloudy from protein deproteinize with 30 mg of solid sulfosalicilic acid for each ml of urine and recentrifuge at 15,000 rpm for 15 minutes. Divide supernatant into two 4 oz bottles for amino acid determinations. Label, cap and store at -20^{o}C.

Appendix III

Microhematocrit Determination

1. Fill each microhematocrit tube 2/3 full with a representative sample of whole blood. Do in duplicate.

2. Plug end, which has not been wet with blood, with a small amount of modelling clay.

3. Place tubes in slots in head of an International micro-capillary centrifuge (Model MB). The plugged end should be against the outer circumference of the head.

4. Centrifuge for 5 minutes.

5. Remove each tube and measure percent packed cells using a microhematocrit reader.

6. Average 2 readings.

Appendix IV

Fluorometric Determination of Kynurenic Acid and Xanthurenic Acid

Equipment:

1. G. K. Turner fluorometer (Model 111)

2. Corning filters:

	Primary Filter (excitation)		Secondary Filter (emission)	
	Corning Number	Wave-length (mμ)	Corning Number	Wave-length (mμ)
Kynurenic Acid	7-60	340	3-75	435
Xanthurenic Acid	7-51	370	3-73	530

For all readings a 1% neutral density filter was inserted with the secondary filter.

3. Ion-exchange columns:

The columns were specially constructed by fusing a 25 cm long pyrex glass tube of 1.2 cm outside diameter to the bottom of a 500 ml round bottom flask. Three cm from the tip the tube was constricted to about one half its diameter, the tip was cut at a 45^{o} angle and fire-polished (fig.5).

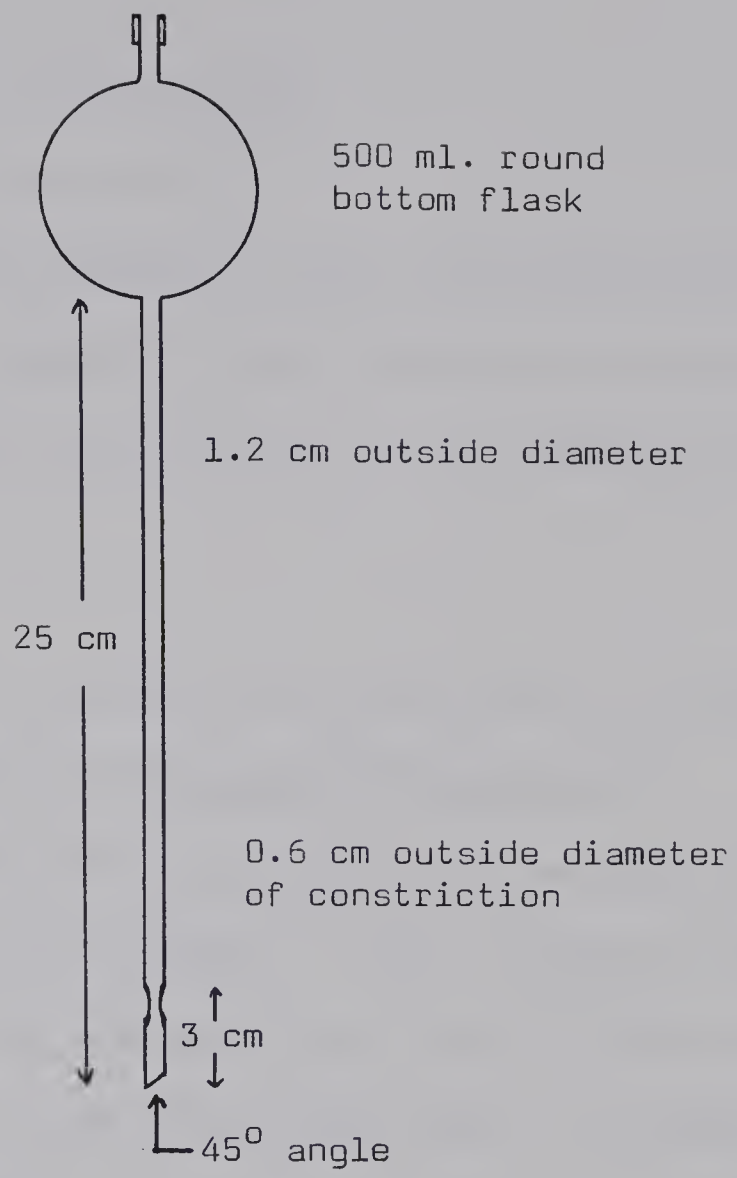

Figure 5 Ion-exchange column used for fluorometric determination of kynurenic acid and xanthurenic acid.

Reagents:

1. Dowex 50 W(H+) X 12, 200-400 mesh.

2. HCl: 0.2N, 0.5N, 1N, and 5N.

3. Buffer: 0.5M KH_2PO_4 adjusted to pH 7.4 with saturated NaOH.

4. NaOH: saturated, allowed to stand several days to facilitate the sedimentation of sodium carbonate which would otherwise interfere with readings.

5. Quinine sulfate:

a) Stock solution containing 15 mg per litre of 0.1N H_2SO_4. This is stable for 3 months at refrigerator temperatures.

b) Working solution containing 0.25 ml of the stock solution per 100 ml of 0.1N H_2SO_4 (0.0375 µg/ml). This is prepared fresh daily.

6. Standard solution of kynurenic acid (3.22×10^{-3}M): 61 mg kynurenic acid dissolved in 100 ml distilled water by the addition of 2 drops concentrated NH_4OH.

7. Standard solution of xanthurenic acid (3.22×10^{-3}M): 66 mg xanthurenic acid dissolved in 100 ml distilled water by the addition of concentrated NH_4OH.

Both standards should be stored at $0^{o}C$ and can be used for 1 month.

Procedure:

1. Wash resin and activate as described by Price and co-workers (13).

2. Column preparation may be done as much as 2 days in advance of the actual operation. Place a small plug of glass wool in the bottom of the column at the point of constriction. Pipet 4.7 to 4.8 ml of

suspended Dowex 50W (H+) resin into the column to give 3.0 $\pm$ 0.1 cm of packed resin. When the resin has settled wash with 50 ml of 5N HCl followed by 100 ml of water. At no time let the water level fall below the upper surface of the resin. Cover the column if not to be used immediately.

3. Preparation of urine samples:

Filter the urine using Whatman number 40 filter paper. The pH of the urine should be acidic; if not add a few drops of concentrated HCl.

a) For the day 1-4 composite use 10% of the volume of urine. Dilute to 240 ml with distilled water and mix well. Measure 120 ml of diluted urine into each of two 250 ml erlenmeyer flasks. To one of the flasks add 1 ml of the standard kynurenic acid solution, 1 ml of the standard xanthurenic solution and 30 ml of 1N HCl. This flask will be used to determine recoveries. To the other flask add 30 ml of 1N HCl.

b) For day 5 and day 6 use 5% of the 24 hour urine volume excreted each day. Dilute each sample to 120 ml with distilled water, transfer each to a separate 250 ml erlenmeyer flask, add 30 ml of 1N HCl to each, and mix well.

In the case of a very concentrated urine or extremely high metabolite levels greater dilution of urine with distilled water will be necessary.

4. Operation of columns:

Add the contents of each flask to a prepared column and allow the sample to pass through the resin. After the sample has run through rinse each flask with 50 ml of 0.2N HCl and pass through the column

followed by 100 ml of 0.5N HCl and then by 15 ml of distilled water. When the water has reached the upper surface of the resin add 396 ml of distilled water to each column. Collect the eluate in 500 ml erlenmeyer flasks each containing 4 ml of 0.5M KH_2PO_4 buffer, pH 7.4. This elution can be allowed to occur overnight if done at refrigerator temperatures.

5. Fluorometric procedure:

a) Prior to the development of fluorescence prepare blank and standard solutions:

To three 500 ml erlenmeyer flasks add:

Constituent (ml)	Flask 1 (Kynurenic Acid)	Flask 2 (Xanthurenic Acid)	Flask 3 (Blank)
Water	395	395	396
0.5M KH_2PO_4	4	4	4
Standard kynurenic acid	1	0	0
Standard xanthurenic acid	0	1	0
Total volume	400	400	400

b) At the time of fluorescence development:

(i) Prepare a fresh working solution of quinine sulfate.

(ii) Prepare dilute standards, samples, and blank in duplicate. Depending on the expected concentration of each of the metabolites in the urine vary the amount of sample used.

For kynurenic acid: Add reagents to 25 X 200 mm pyrex test tubes.

	Constituent (ml)	Standard	Blank	Sample
1) Add	Eluate (from column)	--	--	1-5
	Blank (from Flask 3)	5	6	to 6
	Dilute kynurenic acid (from Flask 1)	1	--	--
2) Immerse in ice				
3) Slowly add				
	Conc. H_2SO_4	4	4	4
4) Mix by gentle swirling				
	Total volume	10	10	10

For xanthurenic acid: Add reagents to 15 ml centrifuge tubes.

	Constituent (ml)	Standard	Blank	Sample
1) Add	Eluate (from column)	--	--	1-5
	Blank (from Flask 3)	4	5	to 5
	Dilute xanthurenic acid (from Flask 2)	1	--	--
	Saturated NaOH	5	5	5
2) Stir with glass rod to mix well				
2) Centrifuge for 10 minutes at 2000 r.p.m. (Sorvall SS-3 Super-speed centrifuge).				
	Total volume	10	10	10

6. Reading of fluorescence:

Transfer the contents of the tubes to matched fluorometer cuvettes for reading. Read the fluorescence in the fluorometer with the range selector positioned at 10X.

a) <u>For kynurenic acid</u>: Set the fluorometer at 20 with quinine sulfate solution. Read blanks, standards, and samples and record. Reset the fluorometer after every 4 to 6 tubes with quinine sulfate because it is much more stable to irradiation than is kynurenic acid.

b) <u>For xanthurenic acid</u>: Set the fluorometer at 25 with quinine sulfate solution. Read and record blanks, standards, and samples as for kynurenic acid.

7. Calculations:

a) μmoles metabolite in 24 hour sample $= \frac{(\text{sample reading} - \text{blank})}{(\text{standard reading} - \text{blank})}$

$$\times \frac{3.22}{\text{eluate used (ml)}} \times \frac{\text{24 hour urine volume (ml)}}{\text{urine volume (ml) before dilution to put on column}}$$

b) % recovery $= \left[\frac{(\text{recovery reading} - \text{blank})}{(\text{standard reading} - \text{blank})} \times \frac{3.22}{\text{recovery eluate used (ml)}} \right]$

$$- \left[\frac{(\text{sample reading} - \text{blank})}{(\text{standard reading} - \text{blank})} \times \frac{3.22}{\text{sample eluate used (ml)}} \right] \times \frac{100}{3.22}$$

c) μmoles metabolite in 24 hour sample when 100% recovery =

$$\mu\text{moles metabolite in 24 hour sample} \times \frac{100}{\%\ \text{recovery}}$$

Appendix V

Fluorometric Determination of N^1-Methylnicotinamide in Urine

Equipment:

1. G. K. Turner fluorometer (Model 111)
2. Corning filters:
   a) Primary - Corning number 7-60
   b) Secondary - Corning number 3-73, Corning number 4-70, and a 1% neutral density filter.

Reagents:

1. Methyl ethyl ketone - Merck reagent grade or equivalent.
2. NaOH - 40%
3. KH_2PO_4 - ACS reagent grade
4. HCl - 5N
5. Quinine sulfate
   a) Stock solution: Make a solution containing 0.06 mg of quinine sulfate/ml of 0.1N H_2SO_4. Store in a brown bottle in the refrigerator and discard if the solution becomes turbid.
   b) Working solution: Add 1 ml of the stock solution to 99 ml of 0.1N H_2SO_4. Prepare fresh daily.
6. Standard solution of N^1-methylnicotinamide
   a) Stock solution: Dissolve 62.7 mg of N^1-methylnicotinamide chloride in 100 ml of 0.3% KH_2PO_4. This solution contains 0.5 mg of N^1-methylnicotinamide/ml. Store in the refrigerator.

b) Working solution: Dilute 1.0 ml of stock standard to 500 ml with distilled water. This solution contains 1.0 µg of N'-methyl-nicotinamide/ml. Prepare fresh daily.

7. Urea - 35% (dissolved in distilled water). Prepare fresh daily. Keep refrigerated until use.

Procedure for the development of fluorescence:

1. Dilute urine samples so that the resulting fluorescence values (after blank correction) range from 8 to 22.

2. To three 25 X 200 mm pyrex test tubes:

	Samples (duplicate)	Blank
Add:		
diluted urine sample	1 ml	1 ml
distilled H_2O	14 ml	14 ml
cold urea	5 ml	5 ml
Mix on a Vortex mixer.		
Add (using a Corning pipettor):		
methyl ethyl ketone	10 ml	-
Mix well on a Vortex mixer.		
At 0 time add (using a Corning pipettor):		
40% NaOH	2 ml	2 ml
Immediately mix well on a Vortex mixer.		
Let stand for 10 minutes.		
Mix well on a Vortex mixer.		
Add (using a Corning pipettor):		
5N HCl	4.5 ml	4.5 ml
Immediately mix well on a Vortex mixer.		

	Samples (duplicate)	Blank
Add (using an automatic syringe):		
methyl ethyl ketone	-	10 ml

Mix well on a Vortex mixer.

Let stand for 20 minutes. Add:

KH_2PO_4	1 gm	1 gm

Mix on Vortex mixer.

Place in water bath at 85°C for 4 minutes. (Do not shake tubes after insertion in bath as loss of reaction product may occur.) Place in ice bath until reaction mixture has cooled to 23 ± 0.5°C.

Using a Pasteur pipet fill approximately 2/3 full matched 12 X 75 mm cuvettes with an aliquot of the lower layer.

Reading of Fluorescence:

1. Warm up fluorometer for at least 15 minutes.

2. Use the appropriate filters. Position the range selector at 1X.

3. Set fluorometer at approximately 55 with quinine sulfate.

4. When the samples have been removed from the water bath for approximately 20 minutes, so that they are all at the same temperature, read the fluorescence of each cuvette. Check instrument with quinine sulfate after every 2 or 3 readings.

Calculations:

$$\text{mg } N^1\text{-methylnicotinamide/24 hours} = \frac{\text{sample reading - blank reading}}{\text{standard reading} \div \text{concentration of standard (µg/tube)}}$$

$$\text{X urine dilution factor X } \frac{\text{urine volume (ml.)}}{1000}$$

Appendix VI

Macro-Kjeldahl Determination of Urinary Nitrogen (39)

Equipment:

Digestion apparatus

Distillation apparatus

800 ml Kjeldahl flasks

500 ml erlenmeyer flasks

1500 ml burette attached to the standard acid

1500 ml burette attached to the standard base

Reagents:

1. C.P. H_2SO_4

2. Kel-Pak (9.9 gm K_2SO_4, 0.41 gm HgO, and 0.08 gm $CuSO_4$)

3. NaOH - 40%. To 40 liters of water add 340 gm of K_2S. With constant stirring slowly add 20 kg of commercial caustic flake.

4. Standard H_2SO_4 - approximately 0.1N. Accurately titrate with Na_2CO_3 (4 decimal places).

5. Boric acid (4%) solution with mixed indicator. Add 0.02 gm methyl red and 0.10 gm bromcresol green in 100 ml of 95% ethanol to 20 litres of 4% boric acid.

6. Granulated zinc.

Procedure:

1. Place duplicate 2 ml urine samples in separate 800 ml Kjeldahl flasks. For each run place duplicate 2 ml samples of distilled water in flasks to be used as reagent blanks.

2. Add 1 Kel-Pak to each Kjeldahl flask.

3. Add 30 ml of concentrated H_2SO_4 from a burette. Rotate the flask as the acid is added to wash any adhering material from the neck of the flask.

4. Place on digestion rack and digest for 35 minutes after the solution becomes clear green. Rotate the digestion flask occasionally during digestion until all the black specks disappear.

5. Cool on a rack in a closed fume cupboard.

6. While the digestion flasks are cooling, prepare distillate collection flasks by running 50 ml of the boric acid solution into corresponding 500 ml erlenmeyer flasks. Place each flask under a condenser tube, taking care that the tip of the condenser extends beneath the surface of the acid. Turn on condenser water.

7. Switch on the distillation hot plates so that they will be hot when the Kjeldahl flasks are connected.

8. To the cooled contents of the Kjeldahl flasks add a few grains of zinc followed by 275 ml of cold tap water. Then, holding the flask at a 60 degree angle and pointed away from the body swirl the contents and carefully run 110 ml of 40% NaOH solution from a burette down the side of the neck so as to form two layers in the flask.

9. Immediately connect the flask to the condenser by inserting the rubber stopper on the connecting bulb into the neck of the flask. Mix the contents by shaking and distill until all the ammonia has passed over into the boric acid. The first 125 to 150 ml of distillate will generally contain all the ammonia.

10. When the distillation is practically completed, lower the receiving flask, turn off the heat and allow about 5 ml more to distill over in order to wash the condenser tip.

11. Under controlled lighting conditions titrate the contents of the erlenmeyer flasks with standard acid, approximately 0.1N, to a grey end point.

Calculations:

$$\text{gm nitrogen/24 hours} = \frac{\text{normality of standard acid X 14}}{\text{sample size (ml)}} \text{ X}$$

$$\frac{\left[\text{titration volume (ml) sample - blank}\right] \text{ X 24-hour urine volume (ml)}}{1000}$$